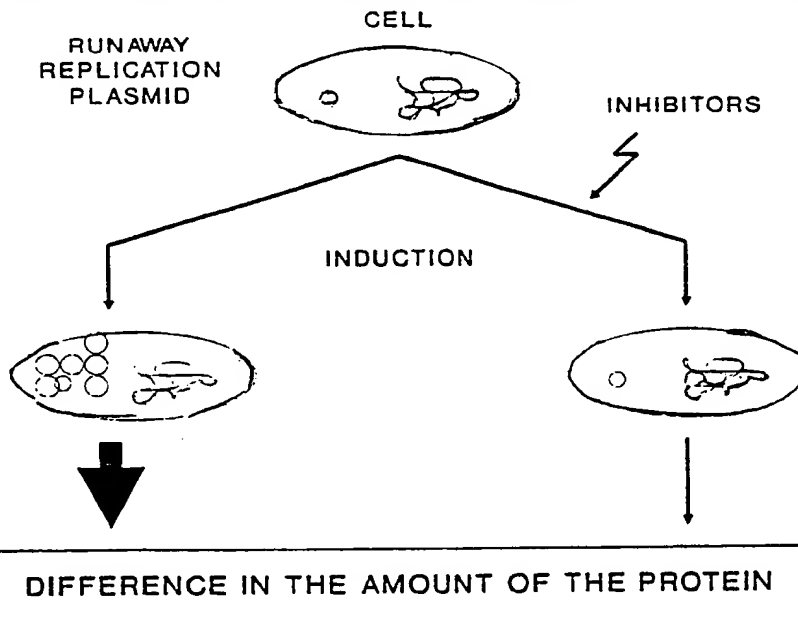




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(54) Title: DETERMINATION OF FACTORS AFFECTING GENE REGULATION AND/OR GENE REPLICATION**(57) Abstract**

The invention relates to a method for determining a factor affecting a cell, which affects directly or indirectly the DNA, RNA and/or proteins of the cell or their synthesis machinery, in which recombinant DNA plasmid is transferred into the cell, and the initial point or points of the reproducing machinery responsible for its replication are subject to an adjustable promoter, which is controllable either by positive or negative feedback; the cell containing the recombinant DNA plasmid is brought into contact with the affecting factor; the affecting factor is allowed to affect the cell containing the recombinant DNA plasmid for a suitable time, after which the promoter adjusting the starting point of the reproducing machinery responsible for the replication of the recombinant DNA plasmid is activated, whereby the copy number of the plasmid starts growing in the cell, unless the affecting factor has not inhibited the replication of the plasmid; the shift of the copy number of the recombinant DNA plasmid in the cell is determined directly or indirectly.

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DETERMINATION OF FACTORS AFFECTING GENE REGULATION AND/OR GENE REPLICATION.

5 Biotests are methods where one uses living cells or
organisms as tools of detection different analytes. Many
of those methods utilize bacterial or yeast cells.
Procaryotic organisms and especially Escherichia coli bac-
terium are very well characterized. Yeast cells are
eucaryotic ones growing anyhow as single cells. The culti-
10 vation of yeast is easier than the cultivation of higher
eucaryotes. Yeast cells grow in simple cultivation mediums
and they need not the addition of complicated growth fac-
tors. The knowledge of yeast is expanding rapidly and com-
prehensive maps of genes are known. Hundreds of specific
15 mutations for both bacteria and yeast are known with which
it is possible to study the activity of specific reactions
and metabolic pathways. For instance with antibiotic sensi-
tive bacterial mutants even trace amounts of antibiotics
cause changes in the metabolism or in the membranes. Using
20 these kind of mutants one is able to develop tests measur-
ing residual antibiotics from biological material very
sensitively. Mutants, whose cell membranes might be porous
for different small molecular weight substances such as
antibiotics. As well bacteria as yeast mutations where the
25 repair mechanisms of their genetic material, DNA, are not
working as well as with the wild-type strains are sensitive
to genotoxic substances. Using different mutant strains
one is able to measure for the presence of antibiotics and
toxic or mutagenic agents.

30 It is rather simple to transfer new characteristics, pro-
teins, into bacterial or yeast cells by genetic engineering
techniques. These proteins, coded by virus, eucaryotic or
procaryotic cell DNA, do not exist naturally in the target
35 organism of gene transfer. This phenomenon expands the
applicability of these organisms in biotests.

The relationship between carcinogenicity and mutagenicity is the basis for using mutagenic agents tests as prescreening tests for carcinogenic agents. The testing for carcinogenicity using test animals is extremely expensive and time-consuming. The testing of mutagenic agents as quick screening for also carcinogenicity has raised much hope and interest. One is thus able to decrease animal based carcinogenicity tests using rapid microbial tests. The basis is the universal genetic code in DNA whose structure and mode of action in each organism is similar.

Most often used test for mutagenic agents is AMES-test (Ames, B.N., McCann, J. and Yamasaki, E. (1975) Mutat. Res. 31, 347) utilizing Salmonella typhimurium bacterium as test organism. With this test one is able to detect the genotoxicity of most mycotoxins, aromatic amines and polycyclic hydrocarbons. However, Ames-test is not able to detect the genotoxicity of carcinogenic metal salts or chlorinated hydrocarbons. In the test the S. typhimurium strains used contain point mutations in the biosynthetic route of the amino acid histidine. As the bacteria are exposed to the action of mutagenic substance, a reversion phenomenon appears in the gene for histidine biosynthesis and the bacterium starts to produce histidine endogenously, thus giving the cell the ability to grow on minimal growth medium containing no added histidine. A pitfall in the test is poor sensitivity and slow performance. In this test all other genotoxic changes such as those acting on enzymes remain undetected. The test is also rather expensive for each particular compound to be tested.

A test for the detection of genotoxic substances based on bioluminescence has been developed (Ulizur, S., Weiser, I. and Yannai, S. (1980) Mut. Res., 74, 113-121). In this method dark mutants of Photobacterium leiognathi and P. fischeri are used. In the presence of genotoxic substances these strains start to emit light. The theoretical back-

ground of the method remains still somewhat obscure. It has been speculated that the effect of removal of a repressor or prevention of its formation combined to a change in chromosomal DNA of the bacterium might trigger the formation of light producing proteins. Different genotoxic substances act with different rate in this test due to the variety of different classes of substances. This test is faster than the Ames-test but is by no means easier to use. The bacteria used in the test should produce light during long and varying periods (30 min to 10 h) of time depending of the substance. The bacteria used are not capable of producing stable light emission, which makes the method somewhat problematic. Due to these facts the method is not easily automatized to be used for routine work when there are lots of specimens to be analyzed. Also being of marine origin the cultivation temperature of the bacteria in this method is rather low, 15°C - it is not known how well the effect of genotoxic substances correlate to the effects on man who's body temperature is at 37°C.

Antibiotics used as medicins against microbial invasion are detected from body fluids in order to study the dosage and penetration of the medicine. The effective therapeutic range of the antibiotic is often rather narrow and the risks due to overdosage might be big. It is also important to measure the presence/concentration of antibiotics from meat and cow milk due to symptoms in allergic people. The cow milk used in cheese production should not contain antibiotics due to the fact that cheese making bacteria are not to able use contaminated milk. The common methods for detecting antimicrobial medicines are microbiological methods performed on agar. A direct method is to measure the inhibition of the growth of sensitive bacterial strains. One can also measure some metabolic parameters such as acid production of a sensitive strain of bacteria using proper colour indicators.

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Typical examples are cylinder, hole or disk methods to make agar diffusion tests. The difference between these tests is only restricted in the way the sample is applied on the agar and also in the way of usage of the bacteria in the test.

Since microbiological methods utilize bacteria or their spores it is the sensitivity of the test bacteria which is of utmost importance. So far one had to make compromises in the choice of a suitable test strain since great sensitivity against antimicrobial agents and other characteristics needed for the test strain have not been common features for the same strain of bacteria.

A major drawback when using microbes in antibiotic residues tests is the slow and unsensitive performance. In these methods one always controls in a way or other the growth of a tester strain one cannot imagine the test to be performed in an hour. This is due to the fact that growth of microbe is a slow phenomenon even at its fastest mode. Also in many cases one uses spores or freeze-dried microbes which makes the tests even more slow to perform.

Antibiotic detection methods based on bioluminescence measurement have been developed. Ulizur (1986, Methods Enzymol., 133, 275-284) describes three different ways to use bioluminescence for the detection of antimicrobial agents: a) lysis-test, b) induction test and c) bacteriophage test. In the first one the lux-genes isolated from Vibrio fischeri produce luciferase protein which in the presence of substrates produces light. The genes have been coupled into a plasmid, which has been transferred to Bacillus subtilis, which is sensitive to antibiotics affecting bacterial membranes such as penicillins and cephalosporins. In the test the B. subtilis containing the lux-genes is grown together with a sample. If there is an antibiotic present the synthesis of cell wall components is prevented and bacteria

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are lyzed, thus yielding a lower light emission level compared to blank.

5 In the induction test one uses dim mutants of P. phosphoreum bacteria, which do not produce light. This as well as other bioluminescence tests developed by Ulizur are based on exploitation of the chromosomal DNA of the target cell. With the induction test one is able to detect antibiotics affecting protein synthesis. When the bacteria are incubated together with compounds binding to DNA they start to produce light ie. protein synthesis is initiated. If there is any antibiotic present affecting the protein synthesis there is a decline in light emission. The amount of antibiotic present is quantitated when compared to blank without antibiotic. With this method one is not able to detect antibiotics affecting DNA synthesis. Also the actual performance of the method is questionable due to the fact that one does not know the basis of the method well. To perform the test it is essential to add minimal salts (such as Ca²⁺ - and Mg²⁺ -ions) which are known to diminish or completely prevent the action of aminoglycosides (streptomycin, kanamycin, neomycin, erythromycin). Also the induction parameters are very strict and if samples contain other antibiotics (for instance nalidixic acid) or other substances triggering light production there might exist problems in the evaluation of the results. The difficulty in this test might be the great number of inducers. Also the amount of bacteria in the test has been claimed to be a critical parameter. If there exists a too high concentration of bacteria the test has to be aerated due to the absolute requirement of oxygen for the bioluminescence reaction in these bacteria. Problems occur when special measuring devices are used and the repetitivity of the assay is affected due to these facts.

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With the bacteriophage test one is able to detect antibiotics affecting DNA synthesis, transcription and translation. Wild-type, light-emitting P. phosphoreum bacteria

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are infected with lytic bacteriophages in this test. In the presence of antibiotic new infective phages cannot be synthesized due to the fact that DNA-, RNA- or protein synthesis is blocked. In the presence of antibiotic the light emission is unchanged compared to initial light level. However if there does not exist any antibiotic the phages rapidly multiply and inactivate the host bacteria thus making it incapable of producing light. Bacteriophage test is difficult to perform since it is necessary to add phages (sometimes with different titers) to the assay mixture and the timing of the addition of antibiotic has to be very careful. In this test same problems exist as with induction test in respect to the composition of assay mixture and the amount of bacteria used in the assay.

One is not able to detect a single antimicrobial chemical or groups of them with microbial methods for antibiotics used till now. Instead, these methods reveal all antibiotics to which the test microbe is sensitive. By changing measuring conditions or by adding enzymes degrading certain compounds one is able to block the effect of some antibiotics. There is a great demand for the detection of heavy metals, toxins or food additives simply and fast is great. At the moment determinations of those compounds have to be performed concentrated in central laboratories, since the devices for their determination are extremely expensive and need special trained personnel to use them. Quick, qualitative tests to be performed in the field could be remarkable filter for those samples which need more sophisticated instrumentation and research. Thus the pressure of central laboratories would be diminished and determination of problematic samples would be fastened.

A commercial "Microtox" test is able to detect toxic substances from environmental specimens. This test is based on the use of light emitting P. phosphoreum bacteria. Sample to be analyzed is incubated together with bacteria and the presence of toxic substance is evaluated from the lowered

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level of light produced by the bacteria when compared to controls. A severe drawback with this test is the need of high salinity (2 %) by the organism, which has been shown to decrease the biological effect of especially heavy metals. Also the measuring temperature of 15°C puts some obstacles. Also several tests utilizing whole animals or animal cell lines have been developed to measure toxic substances, but the pitfall in these methods is the complicated cultivation of cells, slow performance and need for skilled personnel.

Toxic and mutagenic substances should be able to be detected for example from different waters such as from waste-, consuming-, raw- and groundwater and from water for refreshment purposes. Also water needed for industrial processes, food processes as well as raw water needed for pharmaceutical industry are of interest. Samples from ground sediments and air should be able to be detected for their toxicity and mutagenicity. The raw material used in food industry as well as quality control of food stuffs needs great attention. From certain waters one should be able to detect the organic material which could be used for instance to respiration and biosynthetic purposes of microbes contaminating the water. The organic material can be simple sugars, organic acids, peptides or proteins, compounds containing amino or phosphate groups linked to carbon chain etc. There is a need for rapid, non-expensive tests for these kind of compounds since the conventional methods take several days to completion in order to be able to evaluate the quality of water used for various purposes.

The invention described here is based on known and accepted principles on expression of genes and the factors affecting their regulation and on the use of these phenomena in recombinant-DNA organisms such as bacteria and yeast.

Gene technology has made it possible to use bacteria and yeast cells as hosts to produce even such kind of proteins

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that these organisms do not naturally produce. For these purposes several kinds of recombinant-DNA vectors have been prepared; most often then are extrachromosomal plasmids. Recombinant-DNA plasmids can contain several genes and they
5 can proliferate independently. The technique utilizing rec-DNA is the most often used method to transfer foreign genes into new host cells. The gene in question can be joined to a plasmid vector by cutting the gene and plasmid-DNA with specific restriction endonucleases followed by
10 the action of the enzyme DNA ligase which makes the covalent linkage between the gene and the vector. The transfer of hybrid plasmid-DNA into microbial cell can be performed by a transformation protocol whereby the cell wall of the target organism has been made permeable for the DNA. Sev-
15 eral methods exist. When working with eucaryotic cell one uses rec-DNA plasmids which contain parts of DNA of proca-ryotic, virus and eucaryotic origin in suitable combinations. The transfer of DNA into eucaryotic cells is per-
20 formed by for example Ca-precipitation or electroporation techniques.

Many rec-DNA plasmids, where the production of foreign protein has been put under the control of strong promoter, have been developed during the last few years. In each
25 case the goal has been to create as high production of foreign protein as possible in a new organism such as in E. coli. In these cases the production of foreign protein can yield as much as 25 % of total cellular protein (Caulcott & Rhodes, 1986, Trends in Biotech., June, 142-146).
30 If such high amounts of protein are produced it is obvious, that this production is deleterious to the host cell and its metabolism. Due to the harmful effects several plas-mids, where the production of foreign protein has been put under the control of a regulatable promoter, has been de-
35 veloped. The production of protein can here be turned on at the optimal growth phase of the microbe. In these cases the cultivation of the microbe is performed in unstressing conditions until growth has reached the cell density suit-

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able for maximal production of the protein. The protein production is then switched on by adding a chemical to the medium which triggers the production. Also a change in physical parameters such as an increase or decrease in cultivation temperature might in certain cases cause the protein production. There is shown a plasmid pCSS108 (Korpela & Karp, Biotechnol. Lett., 10(6), 1988, 383-388) in Fig. 6, in which the production of bacterial luciferase can be switched on by the addition of chemical coupling agent isopropyl- β -D-thiogalactopyranoside (IPTG) in a cloned E. coli bacterium.

The plasmids commonly used contain one or more resistance factors, with which to select from large population of cells only those which contain the plasmid. The resistance factor helps the cell to survive in circumstances, which are poisonous to other cells. The selecting factor is added to the growth medium to prevent other cells growth except for the one containing the plasmid. The resistance determinant is a gene which codes for a protein which degrades or otherwise inactivates the poisonous factor (which can be for instance an antibiotic) present in the growth medium. Several genes encoding resistance factors are known, the one most often used is the gene coding for β -lactamase which is able to degrade penicillins, or β -lactams which are their derivatives. As the result the poisonous character of penicillin is lost and bacteria can grow. Other commonly used resistance genes are those coding for chloramphenicol acetyltransferase, kanamycin acetyltransferase and tetrahydrofolate reductase. Depending on the type of cells one uses also genes which carry the ability for the cell to grow in the presence of tetracyclin, erythromycin, spectinomycin, streptomycin, sulfonamides, neomycin, thiostrepton, viomycin and colisins. Some resistance factors which eliminate or change the heavy metal present in the medium are also known. Selection pressure in favor to cells containing a plasmid can also be achieved by transferring a gene encoding a function which complements a growth defect, which is lacking

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from the chromosome of the organism. These kind of genes are normally those which code for factors participating in amino acid biosynthesis pathways. In these cases a certain vital amino acid is in short in the growth medium and the cell can not grow unless the gene present in the plasmid produces the enzyme synthesizing the amino acid in question or its intermediate. Also other vital functions result in a beneficial situation for cells containing the plasmid compared to the cells without the plasmid. The plasmid can contain for instance a gene encoding a protein which participates in the formation of the cell wall components or heritable material.

The copy number of various plasmids inside the cell can vary from one to several hundreds, even for over thousand. The most often used plasmid pBR322 has a copy number of about 60 whereas a derivative of it pUC8 has a copy number of about 500. The reason for the high difference between two relative plasmids has been shown to be due to one base pair mutation in the origin of replication (ori) sequence of the plasmid (Chambers et al., 1988, GENE, 68, 139-149). One can artificially raise the copy number of a plasmid at suitable phase of growth by constructing a vector where ori has been put under the control of a strong and regulatable promoter. At present several plasmids are known whose copy number can be artificially shifted up during the growth of microbes. These plasmids are mainly used in industrial processes to produce foreign recombinant proteins in large quantities. Thus the use of these run-away replication vectors for purposes described above does not rule out the possibility of using them in this invention for measuring different agents affecting cell. As examples in this invention we describe different run-away plasmids with which the change in copy number is possible. Those studied and used most to produce foreign proteins are plasmids belonging to series pOU where the origin of replication region has been put under the control of strong and regulatable p_R promoter of phage lambda (Larsen et al., 1984, GENE, 28,

45-54). The p_R promoter of phage lambda is regulated by the repressor protein $cI857$, which is destroyed by heat treatment to 42°C . The production of this protein can be done from a lysogenic phage, from a phage which is conjugated to chromosome of the host cell, from a plasmid where the coding sequence has been introduced or from an other plasmid which belongs to a different incompatibility group. In this context by different incompatibility groups of plasmids are meant plasmids which are able to replicate independently without the presence of another plasmid in the same cell. When the repressor protein has been destroyed, p_R promoter is turned on and without control it starts to produce proteins called copB and repA (originating from low-copy number plasmid R1) as well as transcription products of these and the copA gene. These factors and especially the strong overproduction of repA protein result in enhanced or even uncontrolled production of the plasmid-DNA in E. coli bacterium.

Yeasts as well as bacteria are single cell organisms but yeasts differ from bacteria by being representatives of eucaryotic cells. Compared to higher eucaryotic cells yeasts are far better characterized from the genetical point of view. The genetic maps of Saccharomyces cerevisiae and Schizosaccharomyces bombei are already known in great detail (Petes, 1980, Ann. Rev. Biochem., 49, 845-876). Also powerful methods to transfer genes into yeast are known. Indeed, yeasts are commonly used hosts of rec-DNA.

There exists four types of rec-DNA vectors used with yeasts: integration plasmids (YI_p), episomal vectors (YE_p), replicating vectors (YR_p) and artificial chromosomes. The integrating vectors of yeast can contain DNA originating from bacteria and part(s) of yeast genes. This type of plasmid binds exactly on certain point(s) in the yeast chromosome. The replicating yeast plasmids contain DNA from bacteria, part of yeast DNA and a specific area from yeast chromosome, which is responsible for the replication of the plasmid.

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This area permits the plasmid to replicate as extrachromosomal DNA molecule in the yeast cell. The episomal plasmids contain DNA from bacteria, a yeast gene and a part or the whole 2 micron plasmid of yeast (Hollenberg, 1982, Current Topics in Microbiology and Immunology, 96, 119-144). Artificial chromosomes are linear DNA vectors which are not well suited for expression of heterologous proteins.

A plasmid structure for yeast has been described whose copy-number can be regulated. The centromeres of yeast are needed during the partition of chromosome in mitose and meiose phases. Centromeric DNA (CEN3) has been extracted and transferred under the control of alcohol dehydrogenase promoter (ADH2) which is repressed by glucose. The action of this kind of a plasmid CEN3 can be controlled by the carbon source used to cultivate yeast. When glucose is used as carbon source the ADH2 promoter is repressed and then CEN3 works normally by balancing the plasmid structure (YR_p) during mitoses. If the carbon source is changed in the growth medium the plasmid starts to replicate in the cell and the copy number can rise up to one hundred per one yeast cell (Chlebowicz-Sledziowska, E. & Sledziowska, A., 1985, GENE, 39, 25-31).

The expression vectors used in yeasts contain normally the following strong regulatable promoters: alcohol dehydrogenase isoenzyme I (ADHI) gene promoter, phosphoglycerol kinase (PGK) promoter, repressible acid phosphatase (PHO5) promoter and the promoter for α -factor. ADHI is a cytoplasmic enzyme of yeast, which produces ethanol from acetaldehyde and needs NADH as a cofactor. When yeast cells are cultivated in the presence of glucose there is at least 1% ADHI protein from the total amount of proteins in yeast. The promoter of PGK can be controlled by the carbon source (for example glucose) used, which activates the expression of the protein controlled by the promoter. The expression of PHO5 can be prevented by the addition of inorganic phosphate and again activated by eliminating inorganic phosphate from growth medium. The control of PHO5 happens through

special regulation apparatus, which is formed from PHO2, PHO4, PHO80 and PHO85 gene products (Bosfiana, K.A., 1980, Proc. Natl. Acad. Sci. USA, 77, 6541-6545). Also some mutants (PHO4 and PHO80) are known, which can be activated by a simple change in temperature. These mutant yeast cells grow at 35°C and do not produce acid phosphatase enzyme even if inorganic phosphate does not exist in the medium. If the cultivation temperature is shifted down acidic phosphatase is produced efficiently wheather there is phosphate or not in the medium. This system to control the protein production by a change in the cultivation temperature has been used to produce for instance interferons (Kramer et al., 1984, Proc. Natl. Acad. Sci USA, 81, 367-370).

In order to produce foreign proteins the use of higher eucaryotes as host cells for rec-DNA vectors is rapidly expanding. The wish is to produce proteins of eucaryotic origin in high quantities. In an optimal expression system it would be possible to produce proteins in several different types of cell lines. A fully regulatable expression system for protein production would be an ideal solution. Most often used regulatable promoters work only in certain host cell systems. Often the regulation of these promoters is poor and the expression vectors are based on DNA of tumor producing viruses, thus there exists also certain risks in their uses.

In higher eucaryotes the gene expression can be regulated with the help of following means: simian virus (SV40) T-antigen, metallothionein genes, heat-shock genes, glucocorticoid hormones, DNA methylation or with anti-sense RNA. The antigen produced by SV40 controls its own transcription. T-antigen is produced in high amounts immediately after the virus has infected the target cell and later the T-antigen binds to its own promoter and prevents the transcription. If SV40-vectors are used for cloning the regulation of the T-antigen can be prevented by using a suitable temperature sensitive T-antigen mutant. In these cases T-

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antigen mutants produce T-antigen normally at high temperatures but the production is prevented at room temperature (Rio et al., 1985, Science, 227, 23-28). Metallothioneins are proteins which bind heavy metals. Many eucaryotic cells
5 produce these proteins in the presence of heavy metals. It has been estimated that there is an increase over fifty fold in the production of metallothioneins when cadmium was added to the growth medium to the concentration of 4×10^{-6} molar (Hamer, D.H. & Walling, M.J., 1982, J. Mol. Appl.
10 Genet., 1, 273-288). The protein production induced by cadmium can be further increased by using low Cd^{2+} -content growth media.

Many promoters of heat-shock genes have been shown to be
15 applicable and well regulated in several different cell lines. The regulation of these promoters is performed simply by shifting the growth temperature. The genes are activated to produce proteins at high temperatures, on the other hand at low temperatures the proteins are produced
20 in low amounts or not at all. The best studied case is the heat-shock system of common fruit-fly, Drosophila melanogaster, in which the rise of temperature from 25°C to 37°C causes the ceasing of normal protein production whereas the heat-shock proteins start to emerge. The major and best
25 known heat-shock protein is called hsp70. The regulation mechanisms of the expression of the proteins are not well known. By using heat-shock promoters (hsp70) it has been possible to increase the production of hGH (human growth hormone) up to 1200-fold compared to unactivated cells
30 (Dreano et al., 1986, GENE, 49, 1-8).

In the invention described here advantage is taken of pro-caryotic and eucaryotic organisms, which have been carefully
35 selected and which contain applicable rec-DNA vector constructions. By turning on the synthesis of DNA, RNA or proteins under strict control one is able to measure or detect either directly or indirectly all those factors which affect on the synthesis machineries described above. As

the basis of measurement one can use the protein product encoded by the rec-DNA vector, the marker protein or its activity or the overall metabolic activity. By activating the replication of rec-DNA vector in a controlled fashion one is able to measure the amount of DNA formed also directly using radioactive labels or with flow-cytometric techniques.

One is able to prepare suitable rec-DNA vectors for the measurement of different classes of chemicals depending on what is the target of the chemical. It is possible to quantitate for instance the compounds inhibiting DNA synthesis (nucleotides) and DNA replication as well as those compounds binding to DNA like several cancer drugs by the aid of E. coli bacteria containing runaway-replication type plasmids. The replication of DNA in these kind of plasmids is controlled for instance by an inducible promoter p_{RE} of phage lambda. Thus the DNA synthesis and the replication of plasmid can be triggered at a predetermined point of time and the analytes to be measured can be linked directly to this regulatable and strong biosynthesis of DNA which is not dependable on cell division. If the synthesis or replication of DNA is inhibited the result is seen as the copy number of plasmid is being the same or even decreased compared to the initial stage. In the uninhibited control cells the copy number of plasmid per cell is increasing rapidly. The change in copy number can be measured either directly by measuring the amount of DNA or indirectly by measuring the amount of gene products or the activity coded by the plasmid DNA. It is possible to determine agents that have very different mode of action on the cell with the aid of this kind of a plasmid. This is due to the fact that one can also engineer a gene encoding a marker protein under the control of a strong and regulatable promoter the expression of which is measured in the test. Thus everything affecting DNA, RNA, proteins, their biosynthesis in a way or another, can be measured. If one wants to develop a broad range test which covers agents affecting cell wall,

- nucleic acids, proteins and metabolism an ideal means of detecting these agents is based on this kind of a runaway-replication plasmid. In these cases cells are allowed to replicate after which the promoter regulating the replication is activated and simultaneously or after a certain period also the promoter regulating the gene encoding the marker protein is activated. A vector with similar characteristics can be developed for eucaryotic cells.
- Another kind of approach is to use plasmids whose replication is tied to host cell division. Rec-DNA multicopy plasmids in which the gene encoding the marker protein is under the control of a strong and regulatable promoter can be used to detect agents that affect cell membranes, proteins and metabolism. Agents affecting DNA or cell membranes can be detected with the system if actively dividing cells are used. The multicopy plasmid is synthesized to daughter cells and the system is sensitive to agents affecting DNA. Actively dividing cells are also sensitive to agents affecting the cell membranes. If the strong promoter regulating the expression of the marker protein is activated the system will then also be sensitive to agents affecting the mRNA and protein synthesis.
- As a special application, when genes encoding luciferase are used, there is also a possibility to determine agents that affect energy metabolism. This is due to the fact that the reactions catalyzed by luciferases use energy-rich substances of cells. Agents that can affect the energetic state of the cell on all biosynthetic levels (replication, transcription and translation) or in metabolism, can be determined with the aid of bioluminescence ie. formation of light emission by the cells. A special case is bacterial luciferase, which uses central products of metabolism, NAD(P) and FMNH₂. Another special case is fire-fly and click beetle luciferases, which use central metabolite, ATP, for light production.

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The nature of the invention described here makes it possible to use very different kind of measuring modes for example spectrophotometric, fluorometric, luminometric and visual methods. Spectrophotometric methods can be an alternative when there is a gene cloned into plasmid whose product can be measured by monitoring the change in colour such as β -galactosidase, alkaline phosphatase, amylases, peroxydases, glucuronidases or oxidoreductases. Fluorometric methods utilize fluorescent substrates developed for various enzymes, thus yielding somewhat greater sensitivity compared to spectrophotometric techniques. The luminometric method is performed with the aid of genes encoding either bacterial or beetle luciferases. There exist several luminescent bacterial species such as V. harveyi, V. fischeri, P. leiognathi, P. phosphoreum, Xenorhabdus luminescens etc. Luminescent beetles are for example Luciola mingrellica, Photinus pyralis, Pyrophorus plagiothalamus etc. There exist also several eucaryotic species in the sea which luminesce, such as marine ostracod Vargula hilgendorffii, jellyfish Aequorea victoria, batrachoidid fish Porichthys notatus, pempherid fish Parapriacanthus ransonneti etc., which could be useful in the future for various applications. Here an advantage over spectrophotometric and fluorometric measurement is the extremely sensitive detection of light emission. An important benefit in luminescent methods is the possibility to calibrate internally the measurements by using inside the same cell other genes which encode luciferase emitting a different colour which could be measured with special two wavelenght-detecting apparatus. The other gene can be cloned in the same rec-DNA vector, in an other vector belonging to a different incompatibility group, inserted in the host chromosome, it can be carried in a phage etc. An example is the click beetle luciferases, which emit four different colours the wavelenghts ranging from 547 nm to 593 nm (Wood et al., 1989, Science, 244, 700-702). The other gene resulting in different wavelenght can be put under inducible production system (indicator "gene") or it can be expressed constitutively (internal standard) to compen-

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sate possible secondary effects arising from heterologous samples. The use of simple colour indicator is useful in cases where there is no need for high sensitivity but where the simplicity and fast performance are more important. If the changes in cell metabolism are to be detected one can use for example tetrazolium salts which form a low-solubility formazan colour when reduced. In these cases genes encoding dehydrogenases or oxidoreductases act as mediators of reducing quantities to yield the intense colour of formazan. Also immunological methods (antibodies) coupled to sensitive measuring systems (RIA, FIA) are possible. Use of radioactive labels and flow cytometry in detecting the end point of the test are possible.

15 The method based on the change in copy number of rec-DNA vector:

The cell builds its heritable material, DNA, from deoxyribonucleotides. There can exist also extrachromosomal or episomal DNA as plasmids in the cell. The replication of the plasmids is not directly dependable on the cell proliferation. Each plasmid has its own origin of replication by which to replicate and divide into daughter cells in the course of cell division. However, the plasmid uses the host cell's DNA replication machinery for its own replication.

In Figure 1 is shown a schematic representation of a method based on the change in plasmid copy number in which the cell containing a special plasmid (for example runaway-replication plasmid pCSS123) can be made replicating at a predetermined point of time in a controlled fashion. In the beginning the cell contains only few copies of the plasmid. The agent to be examined is allowed to affect the cell for a suitable period of time after which the replication of the plasmid is commenced. The plasmid will then replicate as much as possible in the presence of the agent. The replication of the plasmid can be triggered by adding a coupling chemical or by physical means like shifting the temperature

high enough for replication to commence. Simultaneously or after triggering the replication the expression of the marker protein can be turned on from the same special plasmid. In this case the degree of the plasmid replication can be directly quantitated by measuring the amount of the marker protein or its activity which is dependent on the copy number of the plasmid inside the cell. This has been described in Figure 1 as an amount of enzyme activity produced by the plasmid encoded gene. This makes it possible to study and measure factors affecting the synthesis of RNA, transcription, translation, cell walls, specific metabolic pathways and enzyme activities as well.

There is shown in Figure 2 a schematic representation on the possibilities to have an effect in the cell with different agents and how they can be coupled to a change in plasmid copy number. The biosynthesis of DNA, RNA and protein are multistep procedures and they need the cooperation of several factors. Each step has both natural and artificial agents which affect the systems either by activating or inhibiting them. For instance nalidixic acid has an effect on the replication of DNA by inhibiting the action of DNA polymerase.

A remarkable thing is that the starting point is few regulatable DNA molecules which can be made to replicate without cell proliferation. This makes it possible to use unproliferating cells for the testing of effectors so that the time used for the assay is not limited by the slow growth and proliferation of cells. The inventiveness of this method is based on controlled multiplication of the plasmid DNA and therefore on a possibility to investigate very large groups of compounds.

In the invention advantage is taken of regulatable promoters and machineries controlled by these promoters such as the increase of a copy number of a plasmid and/or production of a marker protein by the cell at a predetermined phase

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of growth. This context promoters are meant in areas of DNA where the enzyme RNA polymerase can bind, and where a special regulator protein or other molecule can interact as well. Promoters are quantities in the DNA which control the expression of a gene beside or nearby it. Inducible E. coli promoters are for example lac, trp, hybrid promoter tac and p_L and p_R promoters of phage lambda. These promoters differ in respect from each other for instance in strenght and in mode of induction. lac and trp promoters can be induced with chemicals whereas induction of p_L promoter can be started by a simple heat treatment.

Determination of toxic substances using a method where protein biosynthesis is controlled by a regulatable promoter in rec-DNA plasmid:

Another material containing information in cells than DNA is RNA, especially messenger RNA (mRNA). Messenger RNA is synthesized from ribonucleotide triphosphates, which are stored in the cell. Messenger RNA is synthesized according to each gene in the DNA in a special transcription machinery which contains molecules responsible for this action. On the other hand proteins are made according to mRNA-molecule templates using universally accepted principles. RNA synthesis can be switched on very fast as well as the synthesis of a protein coded by the corresponding RNA. In the invention there are used special rec-DNA plasmids which have been prepared so that they can be activated to produce suitably selected proteins in high quantities. In this case a special plasmid has been constructed so that the copy number of the plasmid cannot be selected but it is constant for the plasmid used in certain host cell. Plasmids to be used should be of high copy number and consequently the production of protein would be high. As cells containing these kind of plasmids are treated with agents such as antibiotics affecting mRNA or protein synthesis before induction one could estimate from altered protein production the amount of antibiotic, mode of action or overall presence in the system. The idea of the invention is here applied

to such cases where microbes are exposed to agents which affect biosynthesis routes which are induced specifically and strictly controlled in the presence of these agents. As rec-DNA plasmids are used as described in the invention one is able to get the selected protein production dependent on the agents used.

Remarkable is that one is able to determine agents affecting DNA synthesis and cell membranes. This is possible due to the fact that when a microbe is proliferating it is forced to synthesize these high-copy number plasmids and hence these special plasmids are susceptible to these agents. Actively proliferating cells are especially susceptible to agents acting against cell membranes and their biosynthesis.

There is shown in Figure 3 the practical performance of the invention described above in case where the special plasmid is existing in many copies in the microbe. The microbe is exposed to an agent inhibiting biosynthesis and after a period of time the special plasmids are activated to produce protein, which in this case is an enzyme. The thickness of the arrows shown in the Figure are corresponding to the efficiency of mRNA and protein synthesis and thus also to the efficiency of the agents. In the case where the inhibiting agent has been present the production of the enzyme has remained low. When compared to the case, where affecting agent was not present or there were known amounts of it one is able to perform the measurement either quantitatively or qualitatively.

It is shown in Figure 4 in simpler format those biosynthetic routes which can be affected with the rec-DNA plasmid described above if non-proliferating cells are not used. If one uses actively proliferating cells in the measurement it is also possible to study agents affecting DNA as it is shown in Figure 2. The difference to Figure 1 is the initial amount of plasmid copies in the cell and the possibili-

ty to actificially rise the copy number in non-proliferating cells.

There exist promoters, which can be switched on by suitable treatments. The strenghts of promoters vary a lot and every promoter described in this invention is rather strong. However, one can say that p_L promoter is stronger than lac and trp promoters. The p_L promoter of phage lambda is also much faster ie. the effect of induction is clearly seen much earlier compared to the other two. The rate can be partly explained by the slow incorporation of inducing molecule through cell membranes in the case of lac and trp promoters and further to the effector site inside the cell (for example it can be mentioned the inducer of lac promoter, IPTG). Also the copy number of the plasmid used partly explains the relative differences of the induction. The copy number of a plasmid pCSS112 (see Figure 7) in E. coli is about 60, whereas plasmid pCSS108 (see Figure 6) has a copy number of around 600. The production of bacterial luciferase by the plasmids is controlled in pCSS112 by the p_L promoter of phage lambda and in pCSS108 by the lac promoter of E. coli lactose operon. Both promoters are controlled by certain repressor proteins, which are produced in limited amounts. As the copy number of plasmid in the former case is ten times lower than in the latter case the production of luciferase protein is better shut down ie. repressed. In the latter case the lac promoter leaks due to the relatively low amount of repressor protein and thus the grown level of the protein to be determined or its activity is already on high level. The effect of toxic substances can be shown more effectively when a strong and fast-induced promoter is used to regulate a certain gene or action in a rec-DNA vector.

35 The bacterial strains, plasmids and their construction, methods used in the invention:

As cloning hosts and in toxicity measurements E. coli JM 103 (lac-pro, thi, strA, supE, endA, sbsB15, hsdR4 (F'traD34,

proAB, lacI^qZλM15) (Messing et al., Nucl. Acids Res., 9, 1981, 309-321), MC1061 (cI⁺, araD139, λ(ara-leu)7696, lacX74, galU⁻, galK⁻, hsr⁻, hsm⁺, strA) (Casadaban & Cohen, J. Mol. Biol., 138, 1980, 179-207), BW322 (CGSC, rfa- 5 210::Tn10, thi-1, relA1, spoT1, pyrE) and K-12 (M72 Sm^R-lacZamλbio-uvrB, trpEA2 (Nam7Nam53cI857 HI) (Remaut et al., 1981, GENE, 15, 81-93) and Bacillus subtilis 1A40 (Bacillus Genetic Stock Center, lys-3, metB10, trpC2) were used. Cells were grown on appropriate minimal agar plates and were kept maximally one month at +4°C after which new plates were stroken. The strains were kept also in 15% glycerol at -70°C, wherefrom growth was started through minimal plates. Cells for plasmid extraction were first cultivated in 5 ml of 2xTY medium (16 g Bacto tryptone, 8 g Yeast extract, 8 g NaCl, H₂O ad 1 l, pH 7.4, with appropriate antibiotic) 10 h at 30°C in a shaker after which the cultivation was transferred to a bigger volume for 10 h same medium.

20 There is shown in Figures 5a and 5b the construction of a rec-DNA plasmid pCSS123 (deposited with a DSM number 5119), in Figure 5c the construct of a rec-DNA plasmid pCSS302 and in Figure 5d the construct of a rec-DNA plasmid pCSS305. Plasmid pWH102 (Gupta et al., 1985, Arch. Microbiol., 143, 25 325-329) was cut with the restriction enzymes SalI and PvuII and it was driven in agarose gel electrophoresis. A DNA band of 2300 base pairs (bp) was cut under UV light and the low-gelling temperature agarose was melted at 65°C and ligated with ligase enzyme to a plasmid pEMBL19(-) (Dente 30 et al., 1983, Nucleic Acids Res., 11, 1645-1655) which had been cut with SalI and SmaI. The plasmid obtained was transformed into E. coli JM103 cells using the method described later. A plasmid extraction in mini-scale was performed according to Maniatis et al. (1982, Molecular Cloning: 35 A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor) and the correct constructions were verified with suitable restriction enzyme analysis. Plasmid extraction in large scale was performed according to the same manu-

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al. The plasmid shown in Figure 5a (pCSS111) was cut with the restriction endonuclease PvuII and a DNA-piece of 2400 bp was separated as described earlier. This piece containing bacterial luciferase genes from V. harveyi under lac promoter control was ligated to a plasmid pOU61 (Larsen et al., 1984, GENE, 28, 45-54) cut with the restriction enzyme BamHI which was first filled in with DNA-polymerase Klenow enzyme in a proper buffer system. Ligation mixture was transformed to E. coli JM101 strain as described later and correct plasmid containing colonies were picked by their ability to produce light after visual checking of the plates in dark room. This was performed by adding 5 µl of 10 % decanal on the lid of cultivation plate, which revealed the light producing colonies after few minutes after the aldehyde had penetrated the cells. The plasmid obtained is shown in Figure 5b and was named as pCSS123. An analogous runaway-replication plasmid pCSS302 was constructed as follows: Plasmid pLucGR(tac) (Wood et al., 1989, Science, 244, 700-702) was cut with the restriction endonucleases XhoI and HindIII and filled in with Klenow enzyme. After separation of the fragments on agarose gel a 1800 bp fragment containing the gene encoding green luciferase of click beetle under the control of tac promoter was ligated to plasmid pOU61 which was cut with BamHI and filled in as described above. Ligation mixture was transformed in E. coli JM103 and correct transformants were verified from plasmid minipreparations and the resulting plasmid pCSS302 is shown in Figure 5c. A second analogous runaway-replication plasmid pCSS305 was constructed as follows: Plasmid pCGLS11 (K. Nealson, personal communication and in press) was digested with the restriction enzyme PvuII and a 7 kb fragment containing the genes encoding luciferases α and β subunits of X. luminescens under the control of lac promoter of E. coli were ligated to plasmid pOU61 which was cut with BamHI and filled in as described above. Ligation mixture was transformed in E. coli JM103 and correct transformants were verified from plasmid minipreparations.

The symbols and abbreviations used: amp^r = gene encoding β -lactamase, ori = the origin of replication of the plasmid, luxA and B = genes encoding the subunits of luciferase, lacP = the promoter of the lactose operon of E. coli, F1(IG) the intergenic region of phage F1, MCS = multiple cloning site of pUC18 (Yanisch-Perron et al., 1985, GENE, 33, 103-109), kb = thousand base pairs, repA, copA and cobB = genes encoding proteins responsible for plasmid copy number and partitioning of plasmid in daughter cells, cI857 = the temperature sensitive repressor of phage lambda. The abbreviations of restriction endonucleases used: R = EcoRI, H = HindIII, B = BamHI, S = SalI, P = PvuII, Sa = SacI, K = KpnI, Sm = SmaI, X = XbaI, Ps = PstI, Sp = SphI. B/P = the ligation point BamHI, filled in, PvuII. B/H = ligation point BamHI, filled in, HindIII. B/Xh = ligation point BamHI, filled in, XhoI.

There is shown in Figure 6 a plasmid pCSS108 (Korpela & Karp, Biotechnol. Lett., 10, 1988, 383-388), which is used for production of bacterial luciferase by adding a chemical called IPTG, which triggers protein production by binding to lac repressor protein. The genes encoding luciferase from Vibrio harveyi were transferred from the plasmid pWH102 (Gupta et al., 1985, Arch. Microbiol., 143, 325-329) by cutting the plasmid with the restriction enzyme BamHI. The two pieces obtained were treated with enzyme alkaline phosphatase (CIP) to remove the terminal phosphate groups so that the pieces can not ligate to themselves. A piece of 5000 bp was separated on agarose gel as described previously. This fragment was ligated to a plasmid pEMBL18(+) which had been previously cut with the same enzyme by using T4-DNA ligase. After transformation to E. coli JM103 and overnight incubation of the transformants the cultivation plates were screened for light producing colonies as described previously in dark room. Symbols used are as in Figures 5.

The construction of plasmid pCSS112 (deposited as a DSM number 5120) is shown in Figure 7a. The plasmid contains luciferase genes from V. harveyi and they are under the control of the p_L promoter of phage lambda. The promoter
5 is regulated by the repressor protein $cI857$ of phage lambda, which can be destroyed by short heat treatment in suitable bacterial host such as E. coli K-12 HI trp. Plasmid pWH102 was cut with restriction enzymes SalI and BamHI. Plasmid
10 pPLcAT110 (Stanssens et al, 1985, GENE, 36, 211-233, partly unpublished) was cut with restriction enzymes SalI and BglII. The DNA fragments were separated as described earlier and a 3200 bp piece from plasmid pWH102 and a 2900 bp piece of pPLcAT110 were ligated with the aid of T4-DNA ligase. After transformation into E. coli MC1061 (cI^+)
15 the correct plasmid containing transformant was screened as described above. The plasmid obtained was transformed to E. coli K-12 HI trp host. There is shown in Figure 7b a plasmid pCSS301 which is basically similar as pCSS112 except that instead of gene encoding bacterial luciferase
20 there is a gene encoding green click beetle luciferase (Wood et al., 1989, Science, 244, 700-702). A plasmid pLucGR(tac) containing luciferase gene from click beetle was digested with restriction enzyme BspHI, the cohesive ends were made blunt by a Mung bean nuclease treatment and a DNA
25 fragment of 1643 bp was separated on an agarose gel as described. This fragment was ligated to XbaI - BglII - digested vector pPLcAT110 (described earlier), which was filled in and CIP-treated. The ligation mixture was first transformed to E. coli MC1061 cells and after the correct
30 plasmid was found from plasmid minipreparations it was transformed to E. coli K-12 λ HI λ trp. The symbols used are as in Figures 5, also p_L = leftward promoter of phage lambda., Pv = PvuI, Xb/Bs = ligation point XbaI, filled in, BspHI, Mung Bean treated, Bs/Bg = ligation point BspHI, Mung bean
35 treated, BglII, filled in.

A plasmid pCSS962 was constructed as follows: A shuttle vector p602/22, which can replicate both in E. coli and in

B. subtilis (LeGrice et al., 1987, GENE, 55, 95-103) was cut with restriction enzyme BamHI, filled in with Klenow enzyme and treated with calf intestinal alkaline phosphatase (CIP). The plasmid pLucGR(tac) containing gene encoding green click beetle luciferase was digested with restriction enzyme BspHI, filled in with Klenow enzyme and separated on an agarose gel as described previously, was ligated to the above mentioned shuttle vector. The ligation mixture was transformed into E. coli MC1061 as will be described below and correct plasmid constructions were verified by analyzing the plasmid minipreparations with suitable restriction enzyme analysis. The correct plasmid was co-transformed with a helper plasmid pBL1 (LeGrice et al., 1987, GENE, 55, 95-103) in to B. subtilis 1A40 strain as will be described below. The plasmid and its construction is shown in Figure 7c. The symbols used are: P/O = Promoter-/Operator; ori- = E. coli origin of replication; ori+ = B. subtilis origin of replication; kan = gene encoding kanamycin acetyltransferase; cat = gene encoding chloramphenicol acetyltransferase; T1 = transcriptional terminator.

The competence induction of E. coli strains:
E. coli strains were made competent ie. able to take foreign DNA inside the cell as follows: E. coli was grown overnight in a volume of 5 ml in 2xTY medium and transferred to 100 ml of same medium. After about two hours the optical density as measured at 600 nm was 0.8. The cells were cooled on ice bath and centrifuged 4000xg for 5 min. The cell pellet was suspended in 50 ml of 50 mM CaCl₂ and centrifuged at 3000xg for 5 min at 0°C. The cells were suspended in 4 ml of 50 mM CaCl₂ containing glycerol 15 %. These competent cells were divided in 1 ml aliquotes and they were frozen rapidly in liquid nitrogen and stored at -70°C for later usage.

35

The competence induction of B. subtilis:

B. subtilis 1A40 was grown overnight in 5 ml of 2xTY at 37°C, spun down and suspended in 15 ml of Growth Medium 1

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[(SMS = (NH₄)₂SO₄ 0.2%, K₂HPO₄ 1.4%, KH₂PO₄ 0.6%, Na-citrate 0.1%, MgSO₄ 0.02%)), glucose 0.5%, casamino acids 0.05%, yeast extract 0.06%, MgCl₂ 1.5mM] and grown until the optical density was 1.8 as measured at 600 nm. The culture was then transferred to 150 ml of Growth Medium 2 (SMS, glucose 0.5%, casamino acids 0.01%, yeast extract 0.025%, MgCl₂ 5 mM, Ca(NO₃)₂ 2.5 mM for 90 minutes at 37°C. After centrifugation at 8000 rpm for 5 minutes at room temperature the pellet was suspended in 15 ml of the supernatant. Glycerol was added to 8% and cells were divided into 1 ml aliquotes which were quickly frozen using liquid N₂ and transferred to -70°C.

The transformation of E. coli strains with rec-DNA plasmids: Plasmid-DNA or ligation mixture was added to microcentrifuge tubes 1 to 10 µl on ice bath. To these 250 µl of competent-cells and 26 µl of 10xTMC (100 mM TRIS-HCl, pH 7.4, 100 mM MgCl₂, 100 mM CaCl₂) were added and kept at ice bath for 10 min with occasional careful mixing. A heat shock of two minutes at 42°C was given to cells and one ml of 2xTY was added. The cells were kept thereafter at 30°C for one hour and centrifuged 3 min 8000xg. The supernatant was discarded and cells were suspended in the leftover of supernatant (about 100 µl). The cell suspension was spread on antibiotic selection plates which were kept at 30°C overnight.

The transformation of B. subtilis with rec-DNA plasmids: One ml of frozen competent B. subtilis cells were quickly melted in a 37°C waterbath and they were diluted in 10 ml of SMS Dilution Medium (SMS, glucose 0.5%, MgCl₂ 20 mM, EDTA 1 mM). One ml of diluted cells were mixed with 1 µg of pCSS962 and 1 µg of pBL1 and incubated at 37°C for 30 minutes with shaking. After this cells were plated on 2xTY plates containing 1.0 µg/ml of kanamycin and erythromycin. Plates were incubated at 30°C for 22 hours.

Example 1:

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The change in plasmid copy-number when cells are treated with nalidixic acid:

The plasmid pCSS123 described in the invention is a runaway-replication plasmid, in which the change in copy number
5 can be obtained by shifting the temperature. This has been shown in Figure 8 where it is examined the amount and quality of plasmid-DNA extracted from heat-treated E. coli cells. The effect of an agent known to inhibit DNA replication, nalidixic acid, on cell DNA and especially on plasmid
10 pCSS123 DNA is examined in the Figure.

E. coli pCSS123/JM103 cells were cultivated in 20 ml of 2xTY at 30°C in four Erlenmeyer bottles until the absorbance as measured at 600 nm was 0.3. Nalidixic acid, a known
15 inhibitor of DNA replication, was added to the final concentration of 0, 1, 10 and 100 µg/ml. Immediately parallel samples of 1.5 ml from each bottle was withdrawn to 15 ml tubes and kept at 30°C for 20 min. The tubes were transferred to 42°C for one hour, the bottles were left to 30°C.
20 After this both the tubes and the bottles were kept for an additional hour at 30°C in shaker, after which an extraction of total-DNA was performed from 1.5 ml of cultivations. Cells were centrifuged and the pellets were suspended in 500 µl 50 mM TRIS-HCl, pH 8.0, 50 mM EDTA. Cells were kept
25 at ice bath for 30 min and 50 µl of lysozyme (10 mg/ml) was added and kept at ice bath for 45 min. One hundred µl of STEP solution (0.5% SDS, 50 mM TRIS-HCl, pH 7.5, 0.4 M EDTA) was added and kept at 50°C for 60 min. After this 600 µl of phenol was added and the tubes were gently mixed
30 for 5 min and centrifuged 10 min at 12000xg. Two volumes of absolute ethanol and K-acetate, pH 6.0 to 0,3 M, were added to the supernatant to precipitate the DNA. After 30 min at -70°C the tubes were centrifuged 10 min at 12000xg and the pellet was washed with 500 µl of 70% ethanol, cen-
35 trifuged and the pellets were dried in a vacuum exiccator for 5 min. The dried pellet was dissolved in 50 µl of 50 mM TRIS-HCl, pH 7.5, 1 mM EDTA, 100 µg/ml RNAase A solution and kept at 65°C for 20 min. A conventional agarose gel

electrophoresis analysis was made for the extracted DNA's. This is shown in Figure 8 wherefrom one can see that those samples which were not heat-treated have a DNA content not much changing according to increased nalidixic acid concentration. On the other hand those samples which were heat-treated but did not contain nalidixic acid had a several fold increase in the amount of plasmid DNA. High amounts of nalidixic acid in the presence of cells either heat-treated or not did not change the plasmid content compared to the control. Already 1 ug nalidixic acid was enough to cause a clear decrease in the amount of plasmid-DNA in the heat-treated samples compared to untreated samples.

Example 2:

Determination of toxic substances as measured by the help of light production by cells containing plasmid whose copy-number can be changed.

E. coli pCSS123/JM103 cells grown overnight were diluted 1:1000. Diluted cells in 2xTY were taken (0.5ml) and various amounts of antibiotics or other toxic substances were added. These solutions were incubated 20 min at 30°C. After this the samples were transferred to 42°C for one hour. Each tube was thereafter temperedated to 30°C on waterbath and IPTG was added to 1 mM and n-decanal to 0.01 %. The tubes were transferred to an automated light-gathering device ie. luminometer 1251 (LKB-Wallac, Turku, Finland) whose measuring chamber had been temperedated to 30°C. Measurement of light emission by the cells was done with the auto-mode program so that each tube was automatically measured in every two minutes. The data was collected in the memory of the computer for later analyses. There is shown in Figure 9a the detection of nalidixic acid using E. coli cells cloned with pCSS123. As can be seen from the Figure that already two µg's of nalidixic acid in the measuring conditions can be detected very fast. There is shown in Figure 9b the detection of chloramphenicol using the same approach as in Figure 9a. For clarity reasons only two concentrations of chloramphenicol was compared to

the untreated control. There is shown in Figure 9c the detection of heavy metal cadmium using the same approach as in Figures 9a and 9b.

- 5 Freeze-dried E. coli pCSS123/BW322 were reconstituted with 1.0 ml of 2XTY and 45 µl of this was diluted 1:10 with 2xTY. Five µl of trimethoprim dilutions were added and kept at room temperature for 25 minutes. A heat induction to 42°C was for 25 minutes after which cells were tempered at
- 10 30°C water bath for 10 minutes and measured after the addition of n-decanal to 0.001% for light production with a LKB-Wallac 1250 manual luminometer. Same concentrations of trimethoprim together with reconstituted, freeze-dried cells which were not heat-treated acted as controls. As seen from
- 15 Figure 9d that sensitivity of the assay is very high. There is shown in Figure 9e the detection of ofloxacin using the same approach as in Figure 9d.
- 20 100 µl of reconstituted E. coli pCSS123/BW322 was plated onto the Petri dish. Varying the time the effect of the UV light (254 nm) was tested. After this the samples were transferred to 42°C for 45 min and n-decanal to 0.01% was added. This is shown in Figure 9g.
- 25 E. coli pCSS302/BW322 cells grown overnight were diluted in 2xTY. 90 µl of the diluted cells was taken and the antibiotics were added. The solutions were incubated for 20 min at RT. After this the samples were transferred to 42°C for 45 min. The cells were measured for light production by
- 30 adding 100 µl of solution containing 1 mM D-luciferin and 1mM ATP in 0.1M Na-citrate buffer, pH 5.0. In Figure 9g is shown the detection of ofloxacin and in Figure 9h is shown the detection of citrofloxacin.
- 35 E. coli pCSS305/BW322 cells were used to test the system where no substrate addition was needed to produce light from cells. To 90 µl of the cells in 2xTY 10 µl of the antibiotics was added. Different concentrations of ofloxacin (Figure-

re 9j) and citrofloxacin (Figure 9i) were added and the tubes were kept at RT for 25 min. After this the induction was done by shifting the tubes to 42°C for 45 min. The tubes were loaded in the luminometer for light production measurement.

Example 3:

The detection of antibiotics with a method, where the control of plasmid replication is not possible:

10 In the example a comparison is made between plasmids where the expression of bacterial luciferase genes are controlled by either lac (slow) or p_L promoter (fast). E. coli clones pCSS112/K-12 λ HI λ trp(cI857) were grown overnight in 2xTY medium containing ampicillin 100 μ g/ml. After this a suitable dilution was made in HBSS-buffer or in milk and 500 μ l of this was added to 3 ml luminometer tubes. The tubes were tempered at 30°C and different amounts of various toxic substances were added to tubes. Tubes were kept at 30°C for 20 min, after which the temperature was shifted to 42°C for 10 minutes. Thereafter the tubes were removed to luminometer chamber which had been tempered to 30°C for automated measurement. As a comparison an E. coli JM103 clone containing plasmid pCSS108 was used which was treated similarly without heat-shock step. In this case mRNA and protein synthesis were commenced by adding IPTG to 1 mM. It can be noticed from Figure 10, that when plasmid construction where p_L promoter directs the protein synthesis it is possible to detect toxic substance in much lower concentrations than using a slow and weaker lac promoter.

30 In case of chloramphenicol the kinetics of light production has been shown in Figure 11a when plasmid pCSS112 in E. coli K-12 λ HI λ trp(cI857) strain is used. One can notice from the Figure that differences in the measured activity (light production) are seen from the start of measurement even in as low as 0.1 μ g/ml concentrations in the measuring cuvette.

The detection of antibiotics belonging to penicillin family is of outmost importance since these antibiotics are very

widely used and there do not exist fast methods to detect their presence. There is shown in Figure 11b the determination of ampicillin and also oxytetracyclin and streptomycin using B. subtilis 1A40 cells cloned with luciferase gene from a click beetle. The plasmid used in measurement is shown in Figure 7c. Using this construction the production of luciferase can be turned on in B. subtilis by simple addition of IPTG, which binds to the lac repressor coded by the helper plasmid pBL1 present in the same cell. After binding to the repressor it is not any more able to bind to the DNA region between phage T5 promoter and the luciferase gene thus allowing the expression of luciferase. Cells containing both plasmids were cultivated overnight in 2xTY containing erythromycin (10 µg/ml, to keep pBL1 in the cell) and kanamycin (10 µg/ml, to keep pCSS962 in the cell). A suitable dilution was made and different amounts of antibiotic was added to the cells. After an incubation period of 2 hours at 30°C the tubes were measured for light emission after addition of 1 mM D-luciferin substrate in 0.1 M Na-citrate. As can be seen from Figure as low amount of 0.1 ug/ml of ampicillin and even lower amounts of oxytetracyclin and streptomycin can be detected.

Example 4:

Detection of toxic substances using a method where E. coli contains constant copy-number rec-DNA plasmid and in which p_r promoter of phage lambda controls the biosynthesis of bacterial luciferase and click beetle luciferase:

In the following there is shown some examples on detection of substances affecting other biosynthetic routes and metabolism of cells. The tests have been performed in the same way as those described in previous examples. The goal has been to develop an extremely rapid method, which would anyhow be also very sensitive. Plasmid pCSS112 cloned in the E. coli K-12 strain was used throughout in the following examples.

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The following figures show the effect of each tested substance as measured by light production. As in earlier measurements the presence of inhibiting factors is seen as lowered light production compared to cases where factor
5 has not been present. There is shown in Figure 12 the detection of an antibiotic, rifampicin, which is a known inhibitor of transcription ie. formation of messenger RNA.

As low amount as one μg can be seen very rapidly with the
10 test described in this invention. The effect of oxytetracyclin which binds to 30S ribosomal subunit on the measuring system described here is shown in Figure 13. As in the rifampicin case also here the effect of this antibiotic is strong and easily detected. The effect of sulphite which
15 is a known inhibitor of metabolism and very much used in food processing is shown in Figure 14a. The effect of heavy metal cadmium which is also a known inhibitor of metabolism contaminating soils and water is shown in Figure 14b. These
20 results show that the test system described in this invention is also applicable to the fast determination of metabolic inhibitors. Also it shows that the method can detect the presence of also other agents than antibiotics.

One is able to use different luciferases instead of bacterial luciferase from V. harveyi without losing sensitivity
25 or other performance of the test. There is shown in Figure 15 an analogous measurement as shown in Figures 13 and 14. In the plasmid used in this test (pCSS301) the bacterial luciferase was compensated with click beetle luciferase as
30 described in Figure 7b. The test was done essentially as with bacterial luciferase except that after the cells had been incubated with or without toxic substances 10 minutes at 42°C the cells were measured for light production after
15 minutes temperation time at 30°C by adding 100 μl of
35 solution containing 1 mM D-luciferin, 1 mM ATP in 0.1 M Na-citrate buffer, pH 5.0. Thereafter the light production was measured using a manual luminometer 1250 (LKB-Wallac, Turku, Finland). As can be seen from the Figure the sen-

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sitivity of the method to detect either oxytetracycline or rifampicin is extremely high and comparable to the detection made with bacterial luciferase.

5 Example 5:

The determination of a toxic substance using a method where p_L promoter of phage lambda activates the biosynthetic machinery to produce β -galactosidase:

10 In the previous examples the basis for measurements was the light produced by the bacteria, which phenomenon was due to genes encoding luciferase. As a protein one is able to use whatever protein or peptide, for which there is a method to measure. In this example the gene encoding luciferase has been changed to a gene encoding β -galactosidase of E. coli.
15 The plasmid pPLCAT14 used has been described earlier (Stanssens, P., Remaut, E. & Fiers, W., 1985, GENE, 36, 211-223).

E. coli clones pPLCAT14/K-12 λ HI λ trp were grown overnight in 2xTY medium which was supplemented with ampicillin 100 μ g/ml.
20 After this a suitable dilution was made from bacteria in HBSS buffer and 80 μ l of this was added to glas tubes. Different concentrations of chloramphenicol was added and tubes were kept at room temperature for 15 minutes. After incubation the activation of p_L promoter was performed by
25 shifting the tubes to 42°C for 30 minutes. As a consequence the biosynthesis machinery is activated to produce β -galactosidase coded by the β -galactosidase gene cloned under p_L promoter in plasmid pPLCAT14. After induction toluene is added to tubes to 10 % which makes the cells porous to
30 ONPG chemical. β -galactosidase forms yellow colour which can be measured with a spectrophotometer at 420 nm after reaction with ONPG. After this tubes were centrifuged and measured at 420 nm. There is shown the effect of chloramphenicol in Figure 16 using our method where the protein
35 to be detectd was β -galactosidase and its substrate produce a coloured product.

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Example 6:

Determination of organic content of solution using a method where p_r promoter of phage lambda activates the biosynthetic machinery to produce luciferase:

- 5 E. coli pCSS112/K-12 λ HI λ trp cells were cultivated overnight in 2xTY containing ampicillin (100 μ g/ml). Cells were spun down and washed twice with a HBSS medium (Korpela & Karp, 1988, Biotech. Lett., 10, 383-388) omitting glucose and gelatine but supplemented with tryptophane 0.02%. The cells
- 10 were shaken at this medium for further 4 hours, spun down and suspended in HBSS buffer containing either 0.1% glucose or 0.1% (NH₄)₂SO₄ depending on whether carbon sources or nitrogen sources were evaluated, respectively. A suitable dilution was made from treated cells in minimal salts,
- 15 various amounts of either carbon or nitrogen sources were added and the cells were incubated 10 minutes at 30°C. A heat treatment of 10 minutes at 42°C was given to cells to start the protein synthesis after which the cells were tempered 10 minutes at 30°C before the tubes were loaded
- 20 in the automated luminometer for light production measurements after addition of n-decanal to 0.001%. There is shown in Figure 17a the detection of glucose.

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Table 1. Biochemical targets for drug action:

Cell walls	Inhibitors of protein synthesis	Inhibitors of nucleic acid synthesis
beta-lactams	chloramphenicol	nalidixic acid
cephalosporins	tetracyclines	novobiocin
bacitracin	aminoglycosides	rifamycins
vancomycin	macrolides	phleomycin
polymyxins	erythromycin	mithramycin
gramicidins	lincomycin	actinomycin
valinomycin	puromycin	quinolones

Claims

1. A method for determining a factor affecting a cell, the factor affecting the DNA, the RNA and/or proteins of the cell or the synthesis machineries thereof, characterized in that
- 5
- a) into the cell is transferred a recombinant DNA plasmid, in which the starting point or points of the reproducing mahinery responsible for the replication are subject to an adjustable promoter, which can be controlled either by a positive or a negative feedback;
- 10
- b) the cell containing the recombinant DNA plasmid is brought into contact with the affecting factor;
- 15
- c) the affecting factor is allowed to affect the cell containing the recombinant DNA plasmid for a suitable time, and subsequently the promoter adjusting the starting point of the reproducing machinery responsible for the replication of the recombinant DNA plasmid is activated, whereby the copy number of the plasmid starts growing in the cell, unless the affecting factor has not affected the plasmid in a manner inhibiting the replication;
- 20
- 25
- d) the shift of the copy number of the recombinant DNA plasmid is determined directly or indirectly.
2. A method according to claim 1, characterized in that
- 30
- the recombinant DNA plasmid contains a DNA sequence which encodes one or more selected proteins or a part of it that is essential with regard to its biological activity and the plasmid may comprise one or more DNA sequences, which make the cell resistant to antibiotics, heavy metals and/or
- 35
- toxins.
3. A method according to claim 2, characterized in that the DNA sequence encoding the protein is subject to an

adjustable promoter, which is controlled by a positive or a negative feedback and is adjustable in the implemented microbic cell and is activated simultaneously or at a desired moment subsequent to the activation of the promoter
5 adjusting the starting point of the plasmid replication.

4. A method according to claim 3, characterized in that the factor to be determined is a factor affecting the biosynthesis or replication of the DNA, biosynthesis, transcription or translation of the RNA, the cell membranes,
10 the metabolism or enzymic activity of the recombinant DNA plasmid.

5. A method according to claim 4, characterized in that
15 the factor is aflatoxin, heavy metal, ethidium bromide, nalidixine acid, trimethoprine, fluoroquinolone, aminoglycoside, penicilline, kefalosporine, rifampicin, chloramphenicol, tetracycline, sulphonamide, and the implemented cell is sensitive to the antibiotics to be determined.

20 6. A method according to claim 4 or 5, characterized in that the cell is Escherichia coli and the reproducing machinery responsible for the replication of the recombinant DNA plasmid contained in the bacterium can be accurately
25 adjusted by means of a strong promoter, as for instance lambda P_L and P_R promoters, lac, trp, various hybrid promoters as for instance tac and artificial promoters.

7. A method according to claim 6, characterized in that
30 the recombinant DNA plasmid is pCSS123, pCSS302 or pCSS305.

8. A method for determining a factor affecting the cell, the factor affecting directly or indirectly the DNA, RNA and/or proteins of the cell or their synthesis machineries,
35 characterized in that

a) to the cell is transferred a recombinant DNA plasmid, present in many copies in the cell and the plasmid contains

as a marker protein a protein of a virus, a procaryotic cell or an eucaryotic cell or a DNA sequence encoding a part essential with regard to its biological activity, the expression of which is subject to an adjustable promoter and is controlled either by a negative or a positive feedback;

b) the cell containing the recombinant DNA plasmid is brought into contact with the affecting factor;

c) the affecting factor is allowed to affect the cell containing the recombinant DNA plasmid during a suitable time, after which the adjustable promoter controlling the expression of the marker protein of the recombinant DNA plasmid is activated, whereby the amount of marker protein in the cell starts growing, unless the affecting factor has affected the protein synthesis directly or indirectly;

d) the shift of the marker protein contained in the recombinant DNA plasmid is measured.

9. A method according to claim 1 or 8, characterized in that the cell is a gramnegative or grampositive bacterium belonging to the group Enterobacteriaceae, preferably Escherichia coli or the group Bacillus, preferably Bacillus subtilis.

10. A method according to claim 1-3, 8 or 9, characterized in that the regulating factors of the promoter are produced, either from the chromosomal DNA of the host cell, a plasmid in the cell belonging to another incompatibility class, the same plasmid, a lytic or lysogenic phage or virus or the regulating factors are added from outside the cells, as for instance by adding chemical compounds, altering the temperature or by radiation.

11. A method according to claim 10, characterized in that

the recombinant DNA plasmid can comprise one or more DNA sequences, which make the cell resistant to antibiotics, heavy metals and toxins.

- 5 12. A method according to claim 1 or 8, characterized in that the examined sample is an aerosol in a gaseous, liquid or solid form or the sample is radioactive, ultraviolet or other radiation and of biological or non-biological origin.
- 10 13. A method according to claim 1 or 8, characterized in that the cells are lyophilized and are rehydrated before the determination by a suitable buffer or cultivation medium.
- 15 14. A method according to claim 8, characterized in that the affecting factor is a factor affecting the biosynthesis of the DNA, the biosynthesis, transcription, translation of the RNA, cell membrane or metabolism of the recombinant DNA plasmid, such as mutagenes, antibiotics, heavy metals
20 or toxins.
15. A method according to claim 14, characterized in that the cell is *Bacillus subtilis*.
- 25 16. A method according to claim 15, characterized in that the marker protein in the recombinant DNA plasmid is subject to an adjustable strong promoter like 105, phage T5 lac operator, by saccharose adjustable promotor, and that the marker protein is alfa-amalyse, alkaline phosphatase or
30 luciferase.
17. A method according to claim 14, characterized in that the cell is *Escherichia coli*.
- 35 18. A method according to claim 6 or 17, characterized in that the recombinant DNA plasmid contains a DNA sequence that encodes the protein of the virus, the procaryotic cell or the eucaryotic cell or a part that is essential with

regard to its biological activity and the expression of protein is controllable by means of an adjustable promoter, for instance lac, trp, lambda P and P , various hybride promoters, as for instance tac or artificial promoters.

5

19. A method according to claim 18, characterized in that the protein is luciferase, beta-galactocidase, alkaline phosphatase, proxidase, T4 lysozyme, β 00-glucuronidase, oxidoreductase or pyrophosphatase.

10

20. A method according to claim 19, characterized in that the recombinant DNA plasmid encodes the luciferase of Vibrio harvey or another bacterial luciferase.

15

21. A method accroding to claim 20, characterized in that the recombinant DNA plasmid is plasmid pCSS112, pCSS301 or pCSS962.

20

22. A method according to any of claims 19-21, characterized in that aldehyde is added to the reaction when measuring the amount of expressed luciferase in the battery.

23. A method according to claim 4, 5 or 14, characterized in that the determination is made on milk, serum or water.

AMENDED CLAIMS

[received by the International Bureau
on 17 September 1990 (17.09.90);
original claims 8, 20 and 21 amended; other claims unchanged (5 pages)]

1. A method for determining a factor affecting a cell, the factor affecting the DNA, the RNA and/or proteins of the cell or the synthesis machineries thereof, characterized in that
- 5
- a) into the cell is transferred a recombinant DNA plasmid, in which the starting point or points of the machinery responsible for the replication are subject to a regulat-
10 able promoter, which can be controlled either by a positive or a negative feedback;
- b) the cell containing the recombinant DNA plasmid is
15 brought into contact with the affecting factor;
- c) the affecting factor is allowed to affect the cell containing the recombinant DNA plasmid for a suitable time, and subsequently the promoter regulating the starting point
20 of the machinery responsible for the replication of the recombinant DNA plasmid is activated, whereby the copy number of the plasmid starts to grow in the cell, unless the affecting factor has not affected the plasmid in a manner inhibiting the replication;
- 25
- d) the shift of the copy number of the recombinant DNA plasmid is determined directly or indirectly.
2. A method according to claim 1, characterized in that
30 the recombinant DNA plasmid contains a DNA sequence which encodes one or more selected proteins or a part of it that is essential with regard to its biological activity and the plasmid may comprise one or more DNA sequences, which make the cell resistant to antibiotics, heavy metals and/or
35 toxins.
3. A method according to claim 2, characterized in that the DNA sequence encoding the protein is subject to a regu-

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a) to the cell is transferred a recombinant DNA plasmid, present in many copies in the cell and the plasmid contains as a marker protein a protein of a virus, a procaryotic

cell or an eucaryotic cell or a DNA sequence encoding a part essential with regard to its biological activity, the expression of which is subject to a regulatable promoter and is controlled either by a negative or a positive feed-back;

- b) the cell containing the recombinant DNA plasmid is brought into contact with the affecting factor;
- 10 c) the affecting factor is allowed to affect the cell containing the recombinant DNA plasmid for a suitable time, after which the regulatable promoter controlling the expression of the marker protein of the recombinant DNA plasmid is activated, whereby the amount of marker protein in the
- 15 cell starts to grow, unless the affecting factor has affected the protein synthesis directly or indirectly;

d) the change of the amount/activity of the marker protein encoded by the recombinant DNA plasmid is measured.

- 20 9. A method according to claim 1 or 8, characterized in that the cell is a gramnegative or grampositive bacteria belonging to the group Enterobacteriaceae, preferably Escherichia coli or the group Bacillus, preferably Bacillus
- 25 subtilis.

10. A method according to claim 1-3, 8 or 9, characterized in that the regulating factors of the promoter are produced, either from the chromosomic DNA of the host cell, a plasmid in the cell belonging to another incompatibility class, the same plasmid, a lytic or lysogenic phage or virus or the regulating factors are added from outside the cells, as for instance by adding chemical compounds, altering the temperature or by radiation.

- 35 11. A method according to claim 10, characterized in that the recombinant DNA plasmid can comprise one or more DNA

sequences, which make the cell resistant to antibiotics, heavy metals and toxins.

12. A method according to claim 1 or 8, characterized in that the examined sample is an aerosol in a gaseous, liquid or solid form or the sample is radioactive, ultraviolet or other radiation and of biological or non-biological origin.

13. A method according to claim 1 or 8, characterized in that the cells are lyophilized and are rehydrated before the determination by a suitable liquid or cultivation medium.

14. A method according to claim 8, characterized in that the affecting factor is a factor affecting the biosynthesis of the DNA, the biosynthesis of RNA, transcription, translation, cell membranes or metabolism of the cell containing recombinant DNA plasmid, such as mutagens, antibiotics, heavy metals or toxins.

20

15. A method according to claim 14, characterized in that the cell is Bacillus subtilis.

16. A method according to claim 15, characterized in that the marker protein in the recombinant DNA plasmid is subject to a regulatable strong promoter like Φ 105, phage T5 promoter controlled by lac operator, or saccharose regulatable promoter, and that the marker protein is alfa-amylase, alkaline phosphatase, β -galactosidase, luciferase, peroxidase, T4 lysozyme, β -glucuronidase, oxidoreductase or pyrophosphatase.

30

17. A method according to claim 14, characterized in that the cell is Escherichia coli.

35

18. A method according to claim 6 or 17, characterized in that the recombinant DNA plasmid contains a DNA sequence that encodes the protein of the virus, the procaryotic cell

or the eucaryotic cell or a part that is essential with regard to its biological activity and the expression of protein is controllable by means of a regulatable promoter, for instance lac, trp, lambda P_R and P_L, various hybrid
5 promoters, as for instance tac, or artificial promoters.

19. A method according to claim 18, characterized in that the protein is luciferase, β -galactosidase, alkaline phosphatase, peroxidase, T4 lysozyme, β -glucuronidase, oxido-
10 reductase or pyrophosphatase.

20. A method according to claim 16 or 19, characterized in that the recombinant DNA plasmid encodes the luciferase of Vibrio harveyi or another bacterial luciferase, or beetle
15 luciferase.

21. A method according to claim 20, characterized in that the recombinant DNA plasmid is plasmid pCSS112, pCSS301 or pCSS962.
20

22. A method according to any of claims 19-21, characterized in that aldehyde is added to the reaction when measuring the amount/activity of expressed luciferase in the bacteria.

23. A method according to claim 4, 5 or 14, characterized in that the determination is made on milk, serum or water.
25

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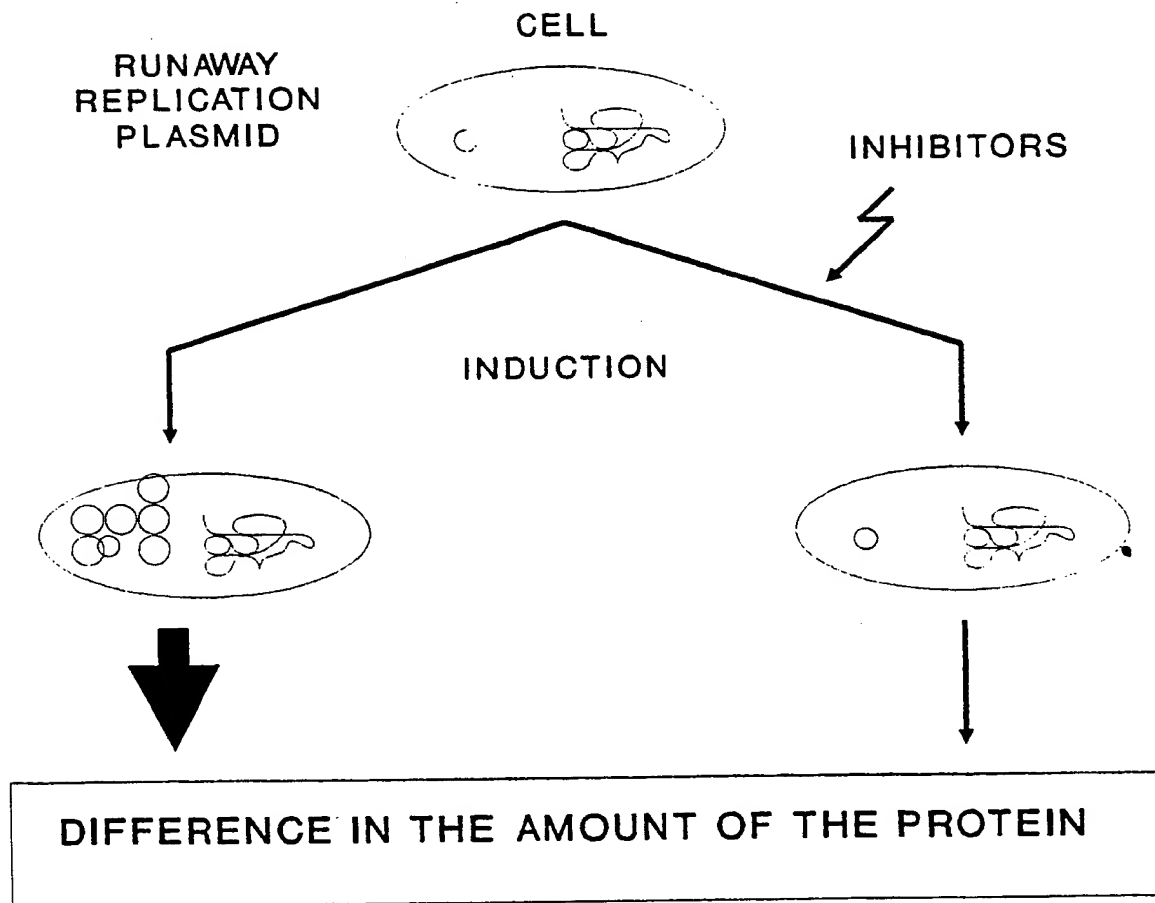


Figure 1

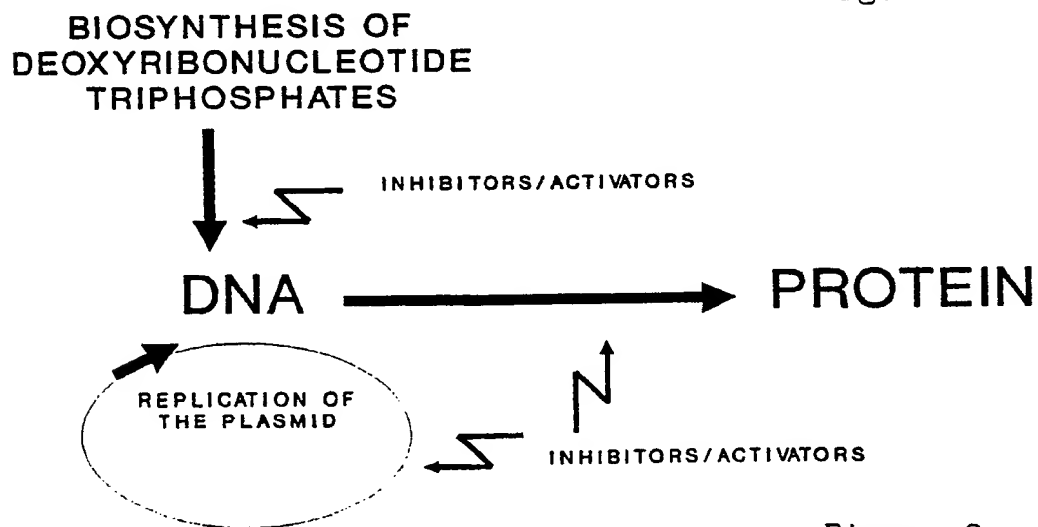


Figure 2

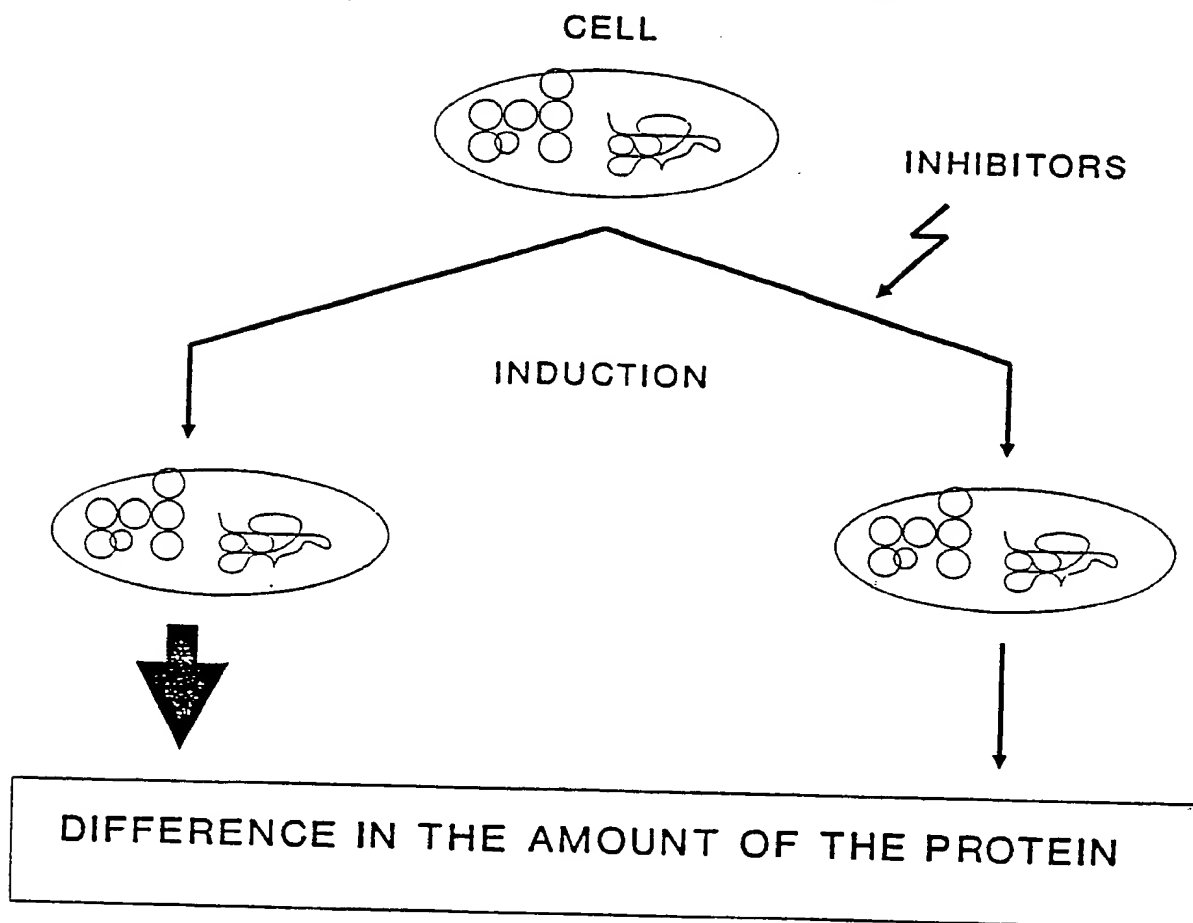


Figure 3

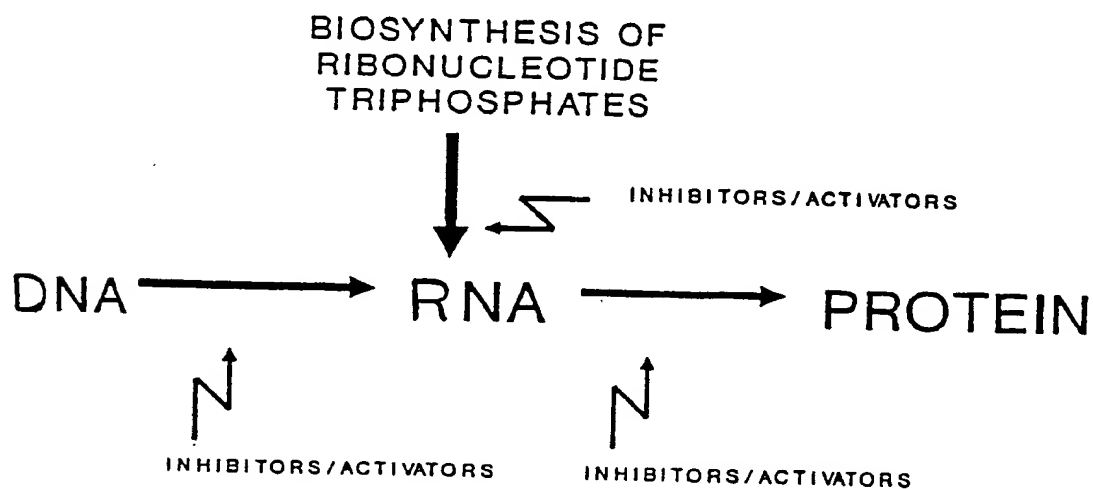


Figure 4

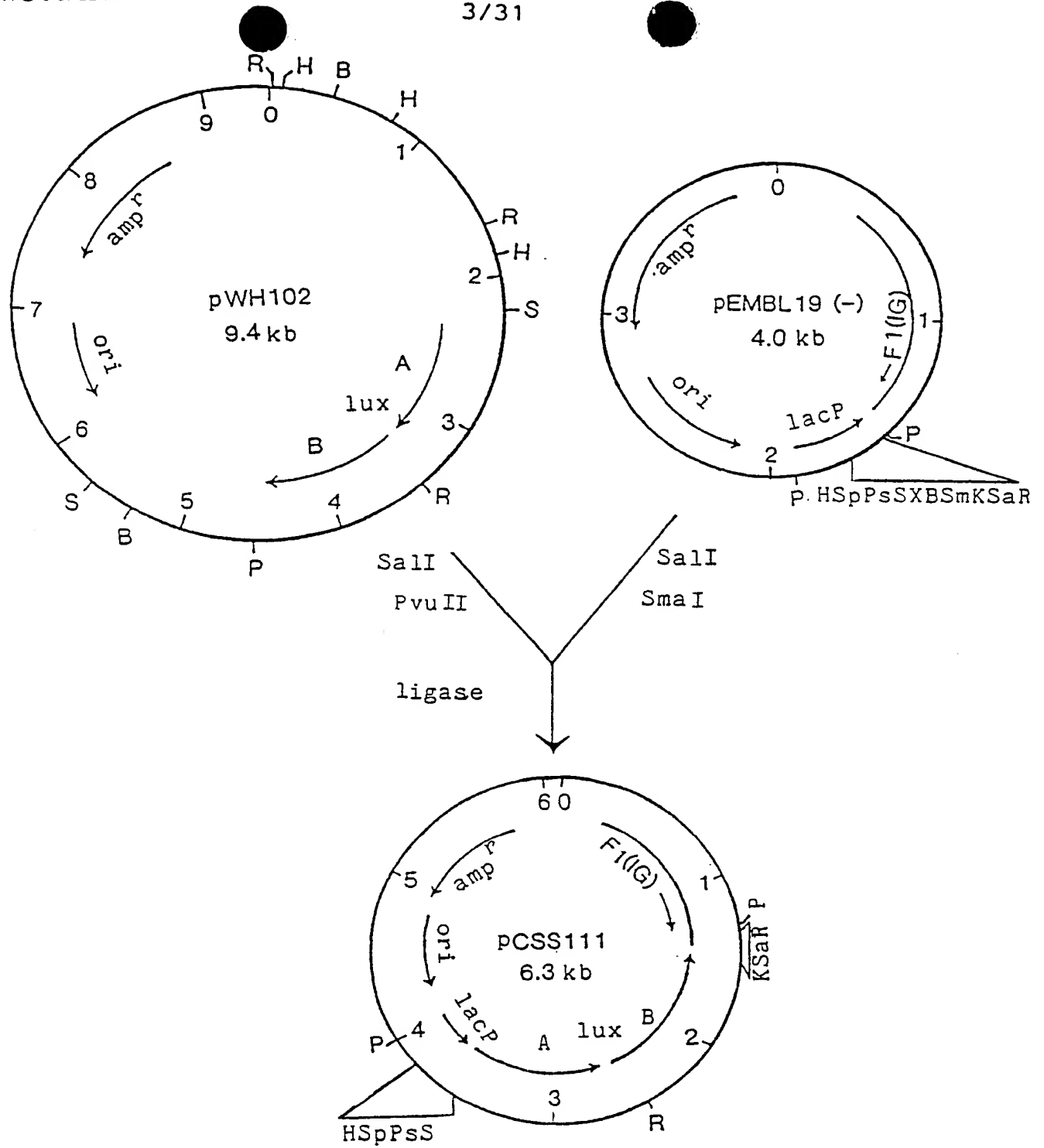


Figure 5a

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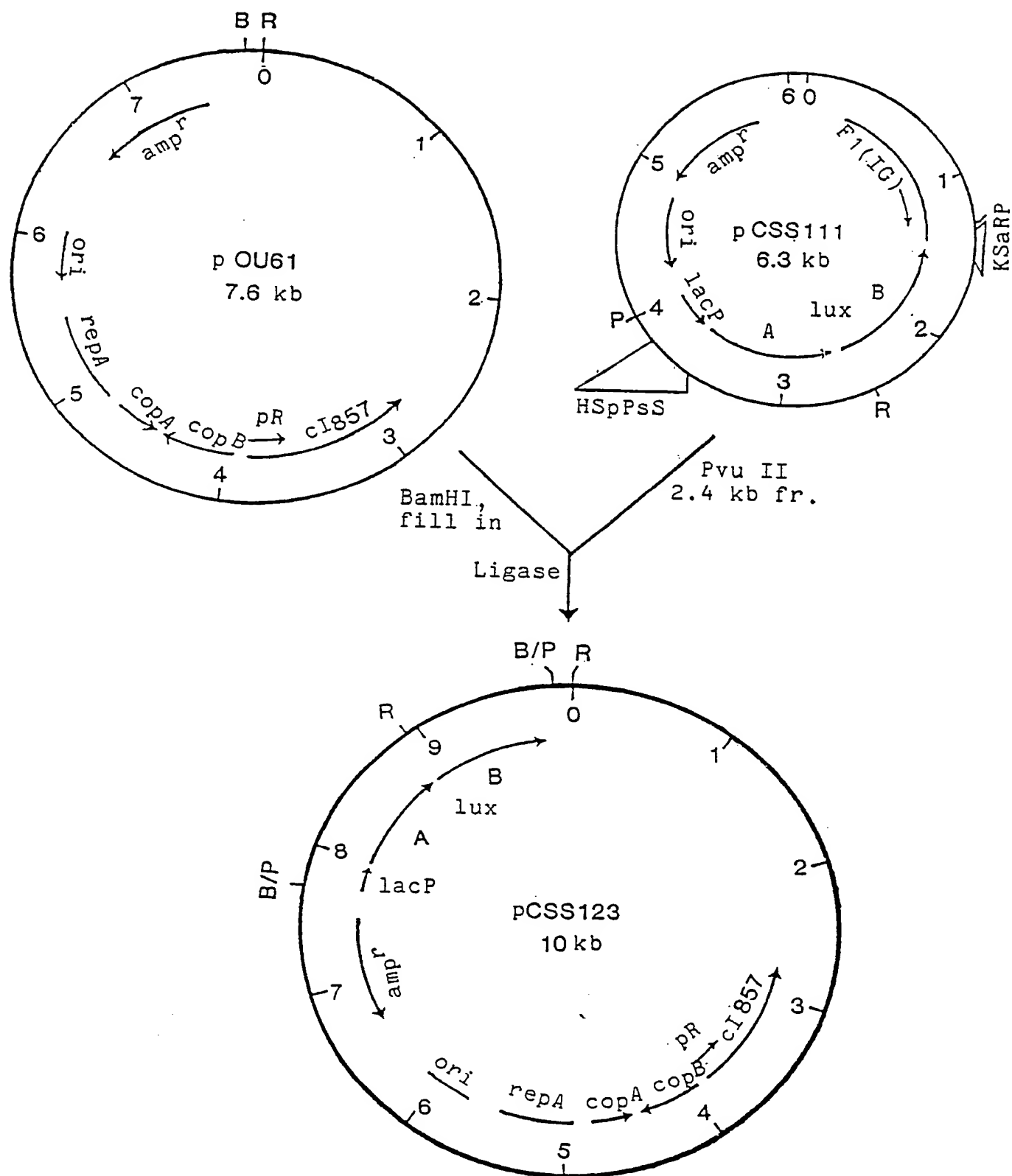


Figure 5b

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pCSS302

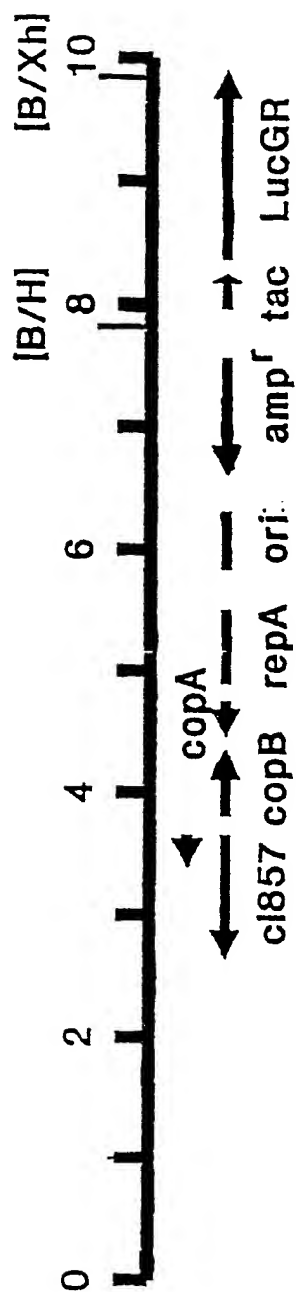
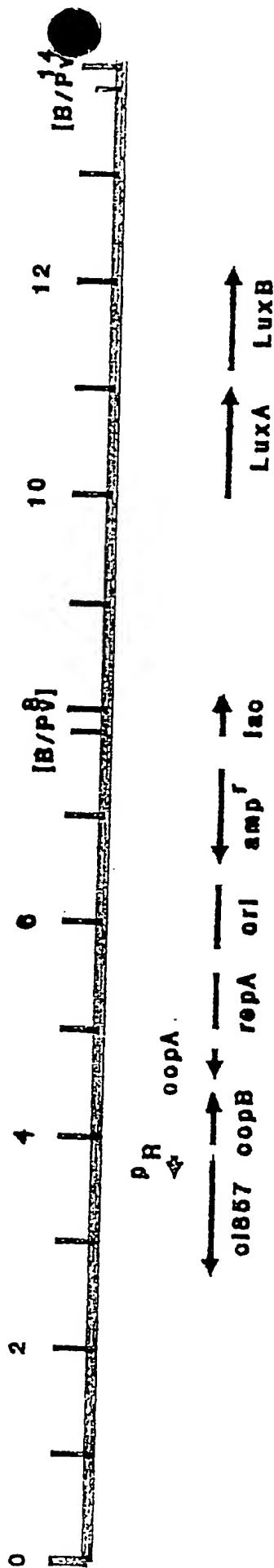


Figure 5c

pCSS305



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partial lux operon of *X. luminescens*

Figure 5d

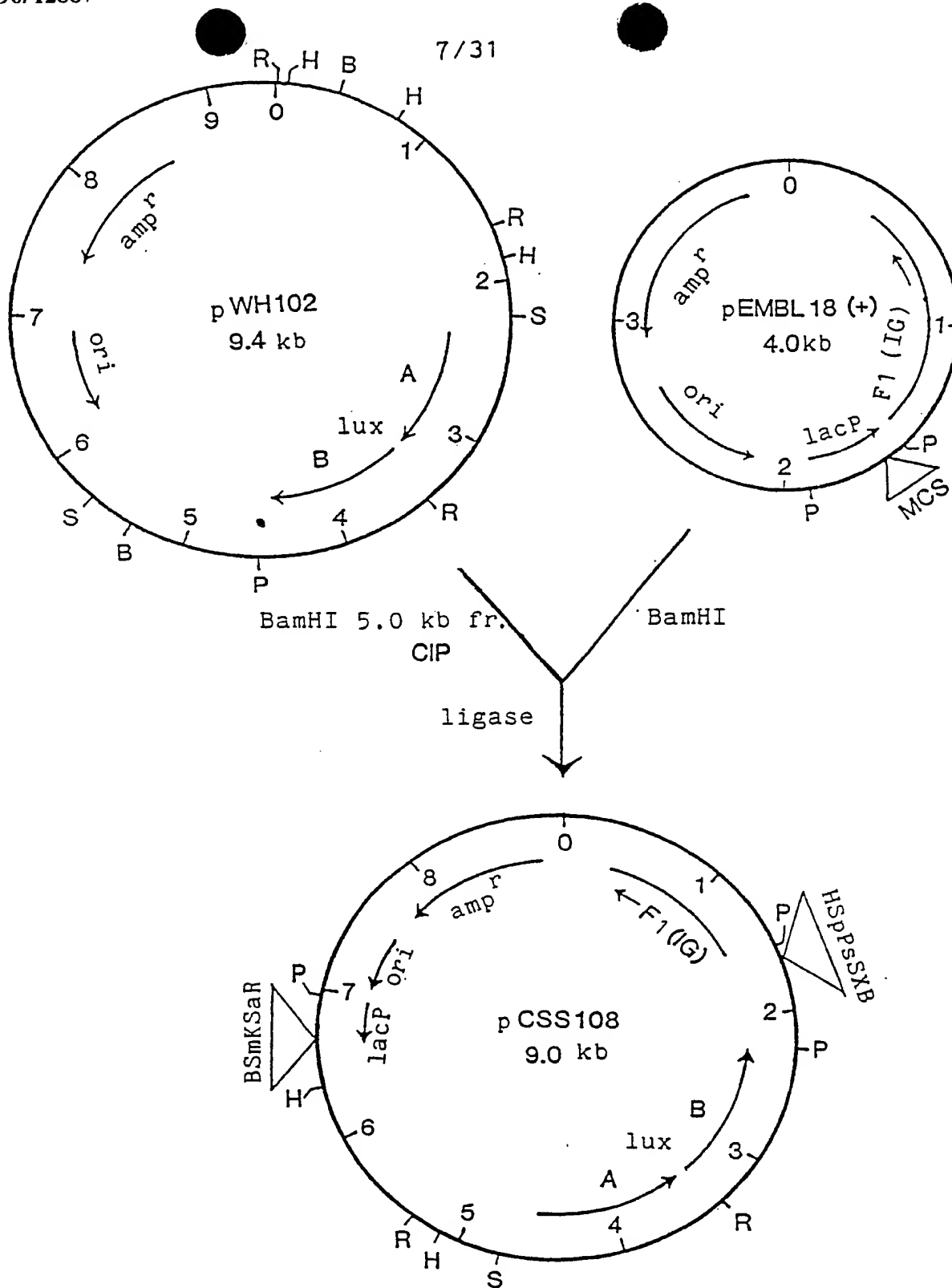


Figure 6

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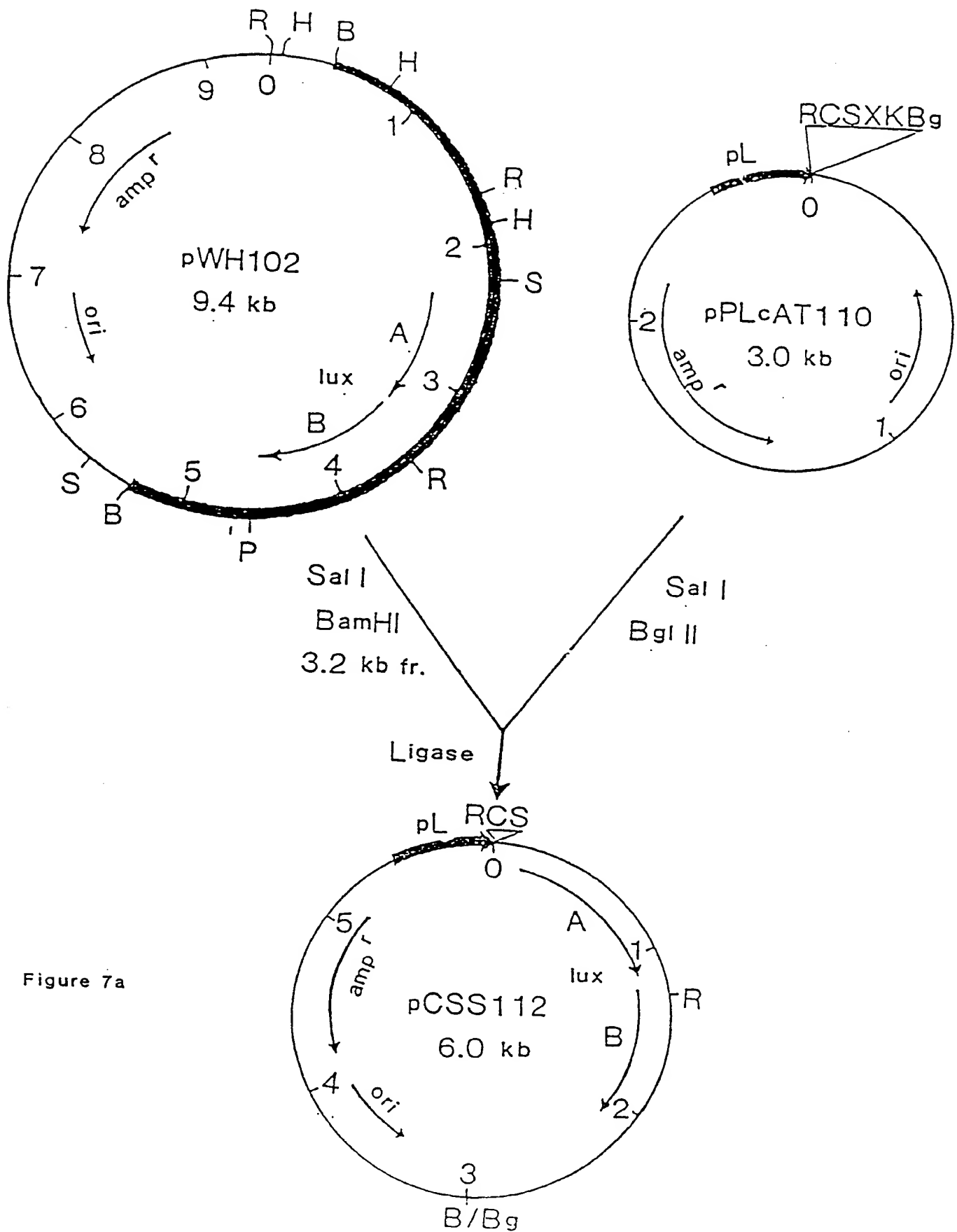


Figure 7a

pCSS301

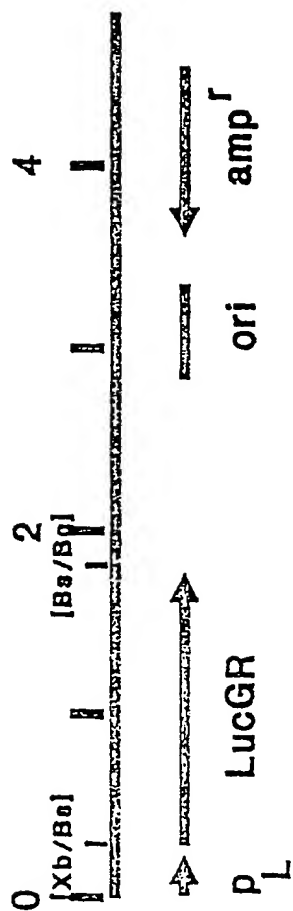


Figure 7b

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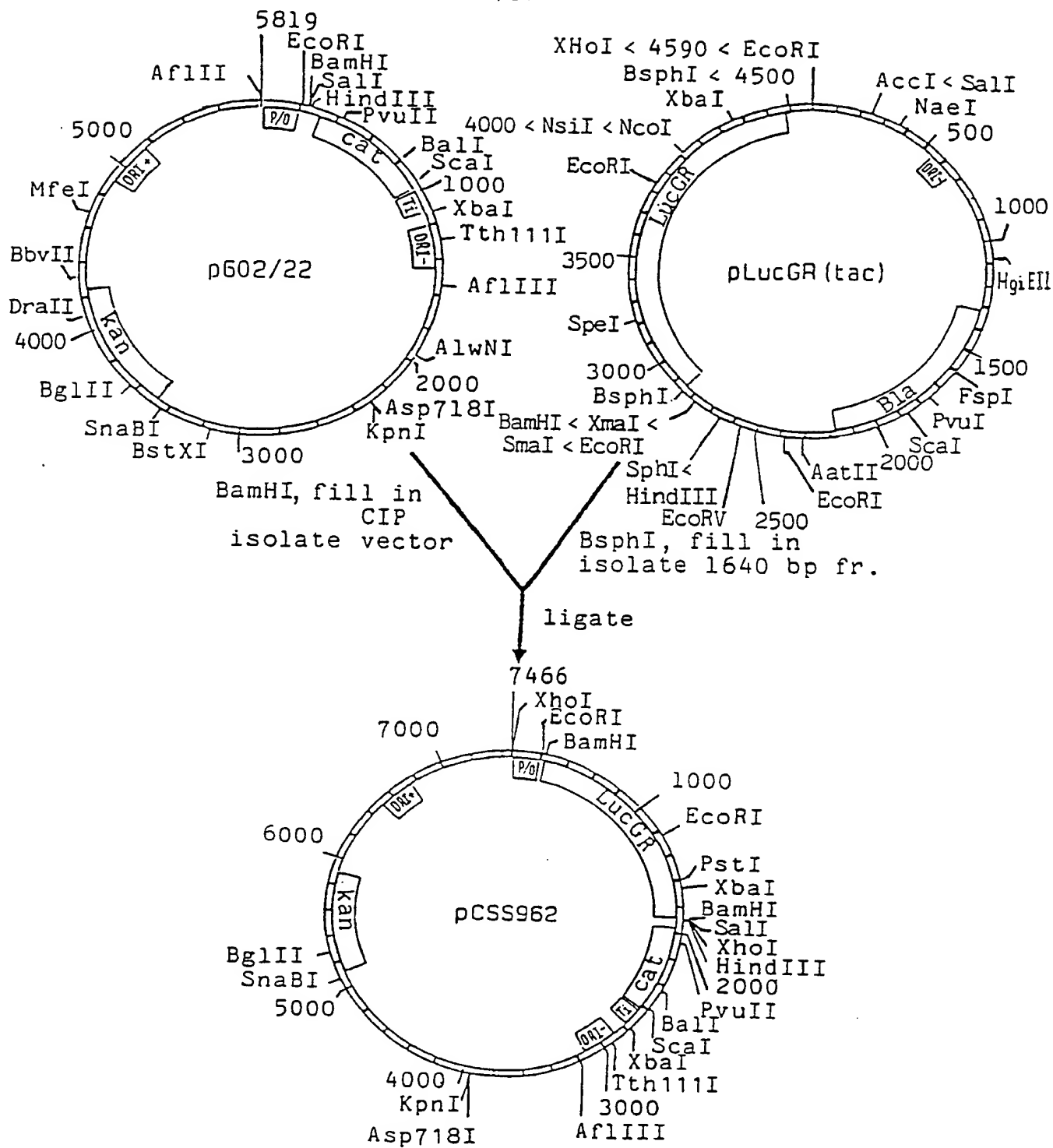


Figure 7c

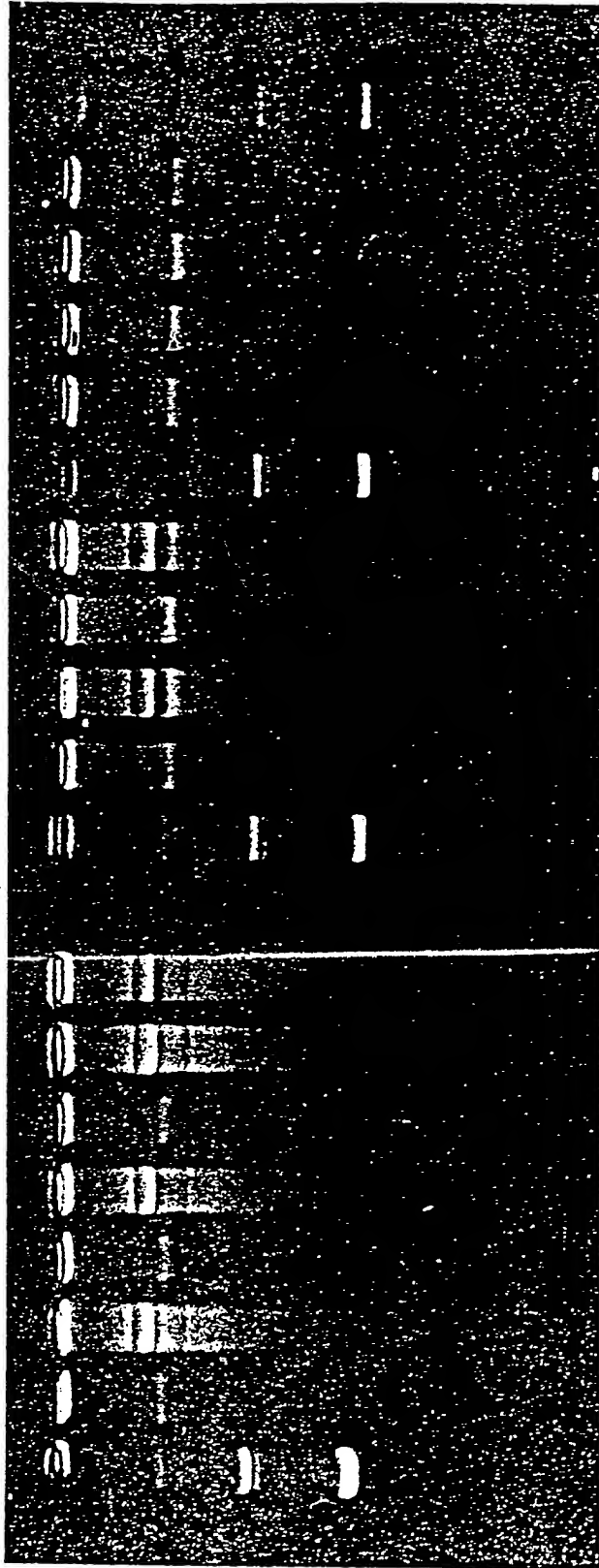
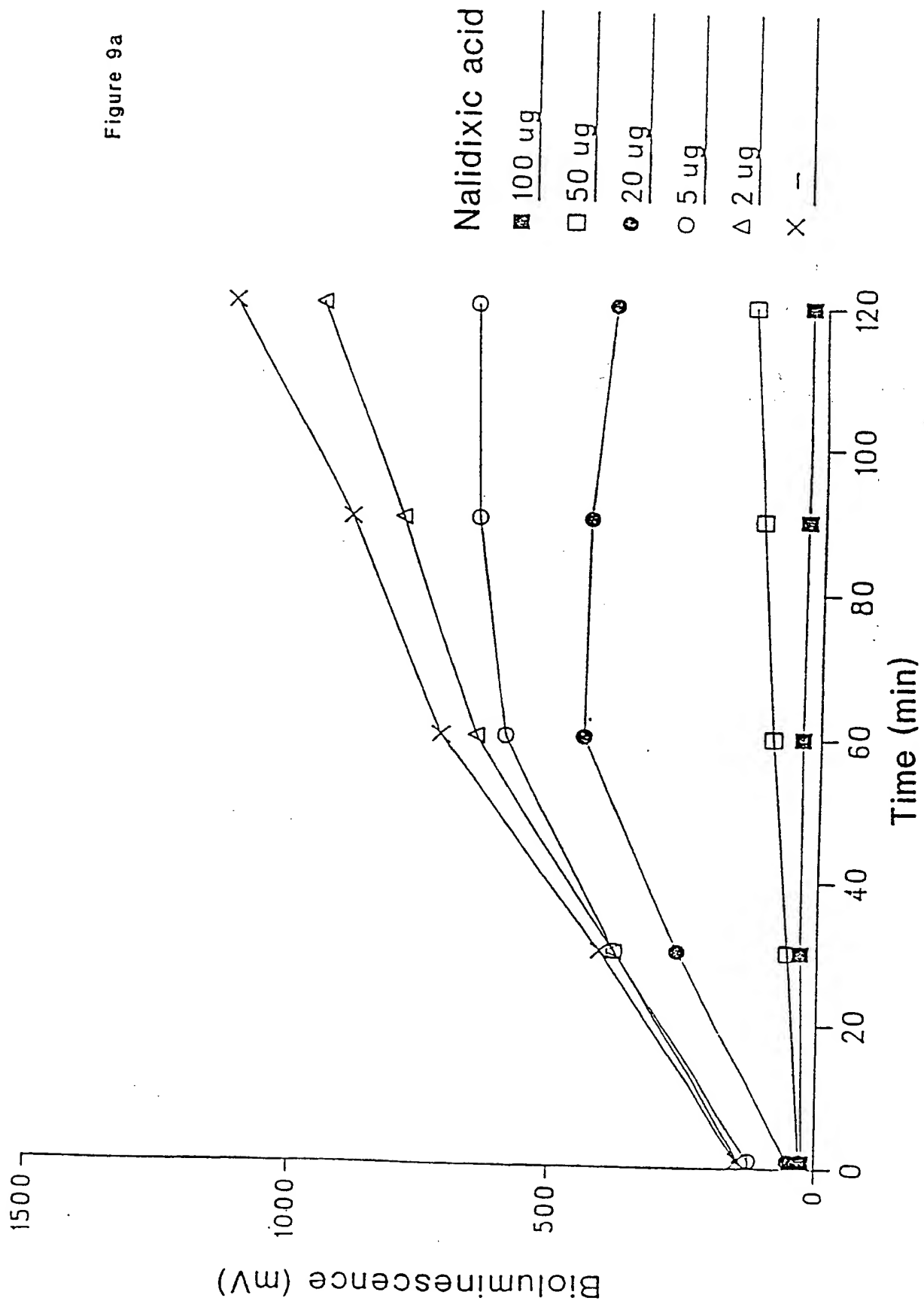
[illegible]

Figure 8

K1. Controlplasmid pUC19 800 ng
K2. 400 ng
K3. 200 ng
K4. 80 ng
O. uninduced pCSS123, nalidixic acid 0 ug/ml
O ind, induced
1. uninduced pCSS123, nalidixic acid 1 ug/ml
1 ind, induced
10. uninduced pCSS123, nalidixic acid 10 ug/ml
10 ind, induced
100. uninduced pCSS123, nalidixic acid 100 ug/ml
100 ind, induced

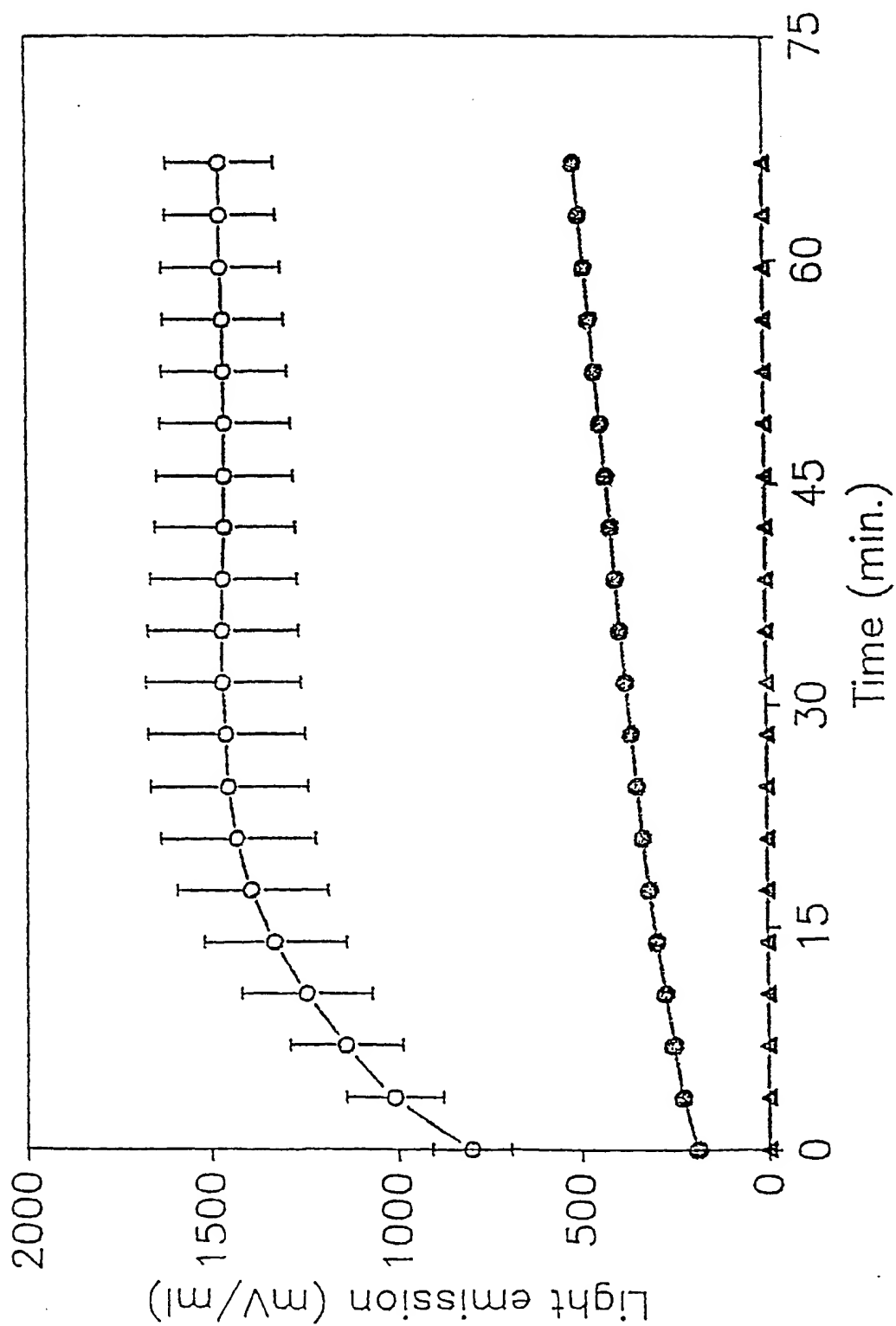
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Figure 9a



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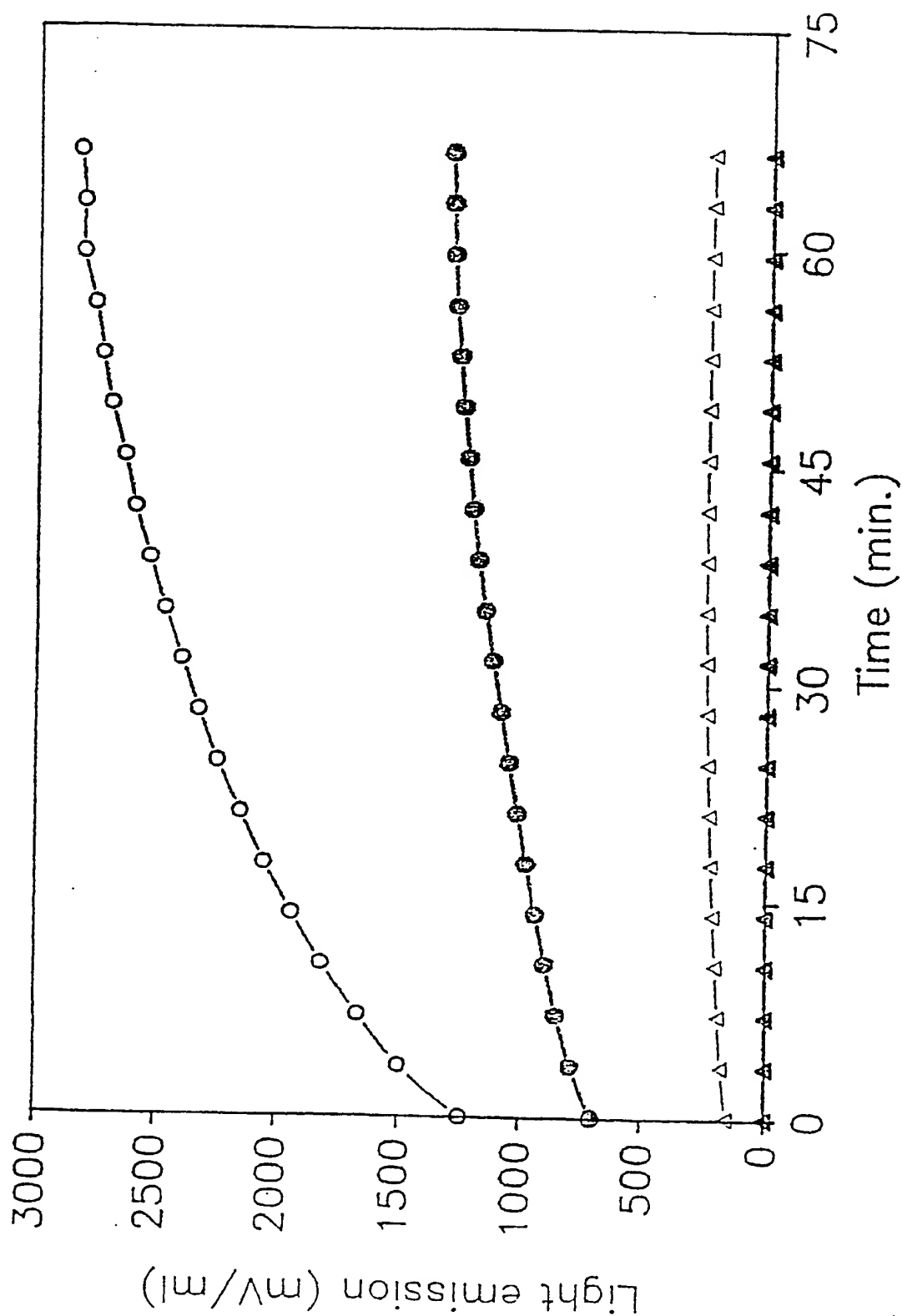
Figure 9b



o—o no Cm, o—o 1.0 mg/l Cm, Δ—Δ 5.0 mg/l Cm, Δ—Δ 10.0 mg/l Cm

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Figure 9c



E.coli pCSS123/BW322(Iyophilized)
Antibiotic: Trimethoprim

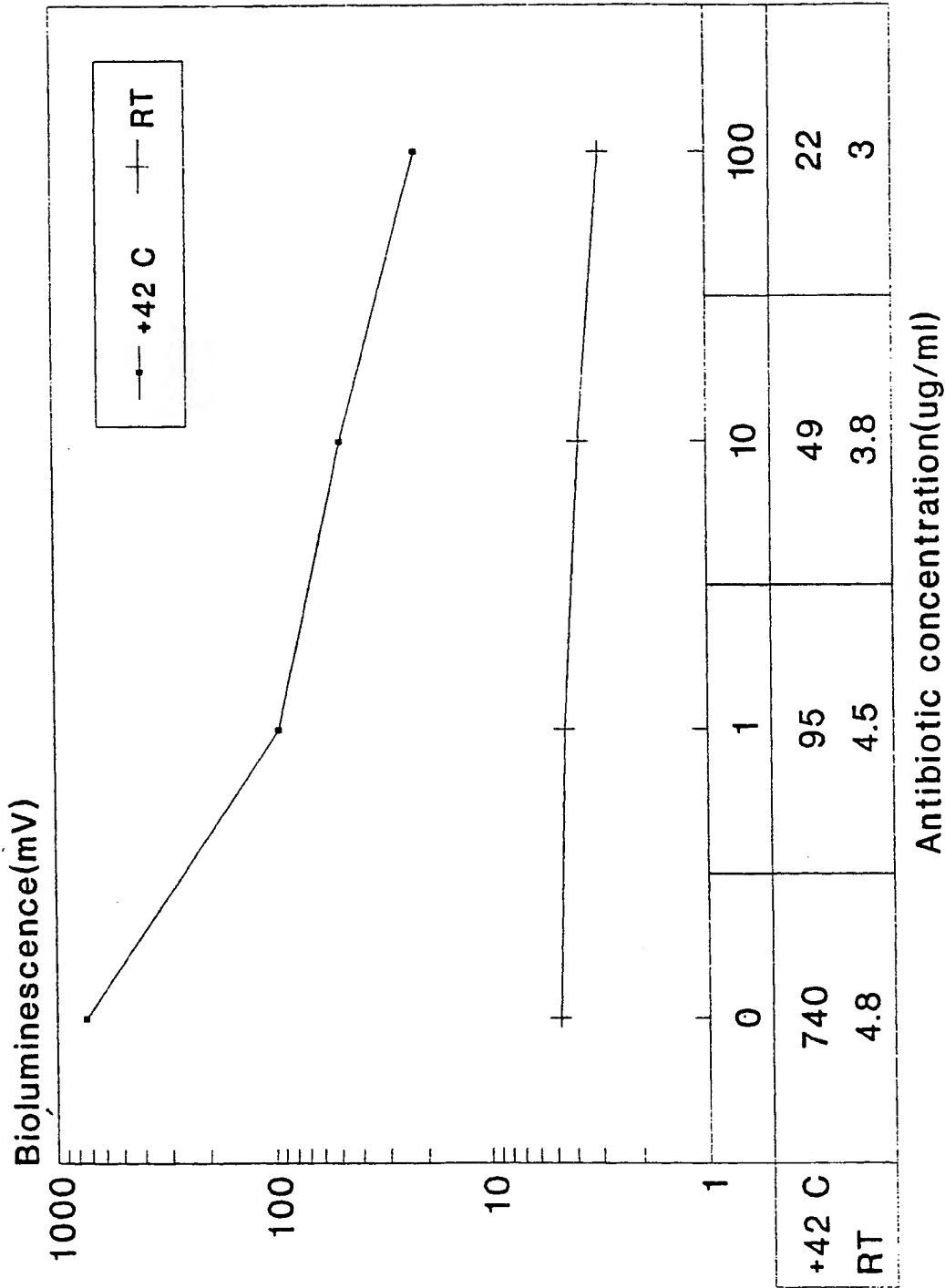


Figure 9d

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E.coli pCSS123/BW322(lyophilized)
Antibiotic: Ofloxacin

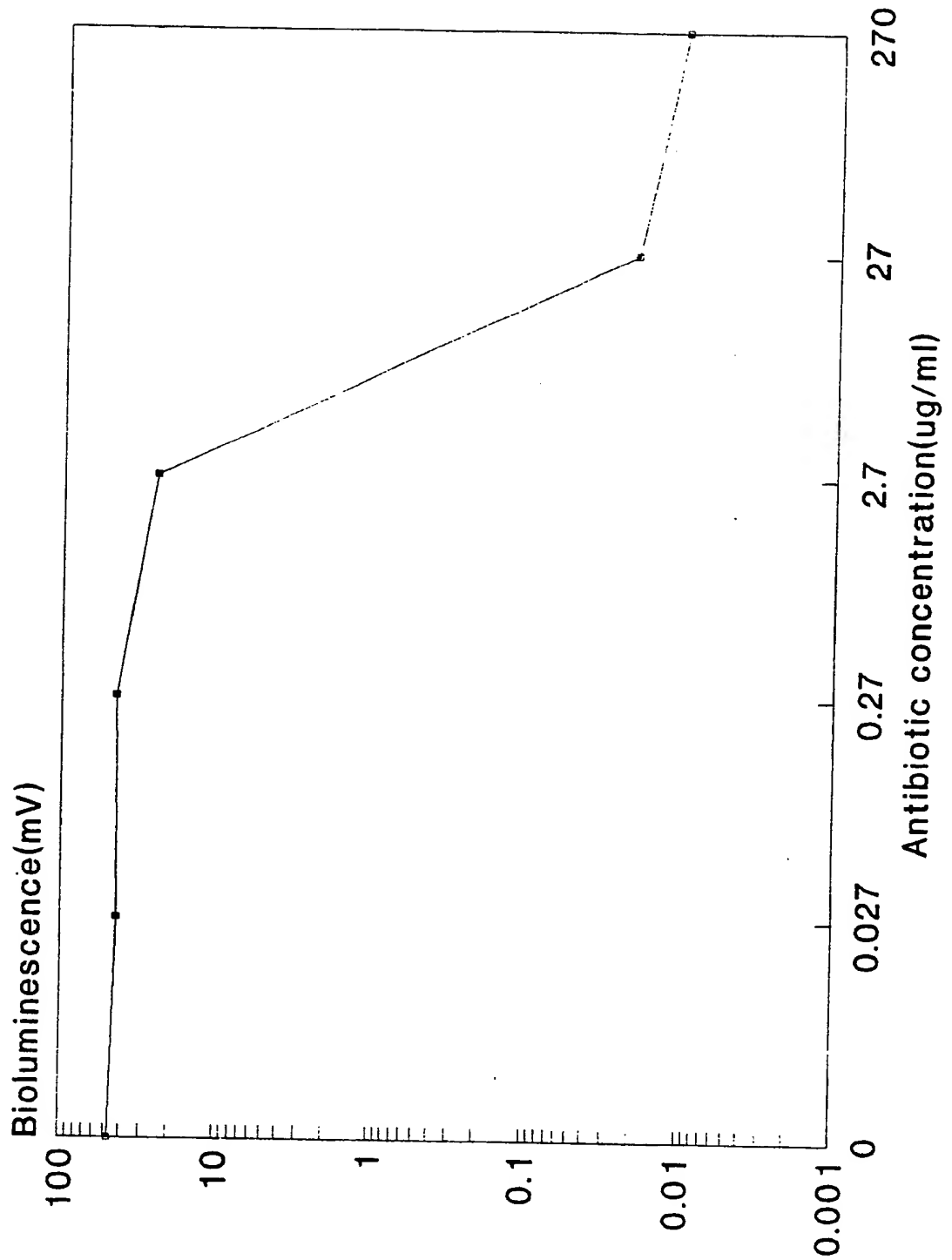


Figure 9e

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E.coli pCSS123/BW322(Iyophilized)
UV-light (254nm)

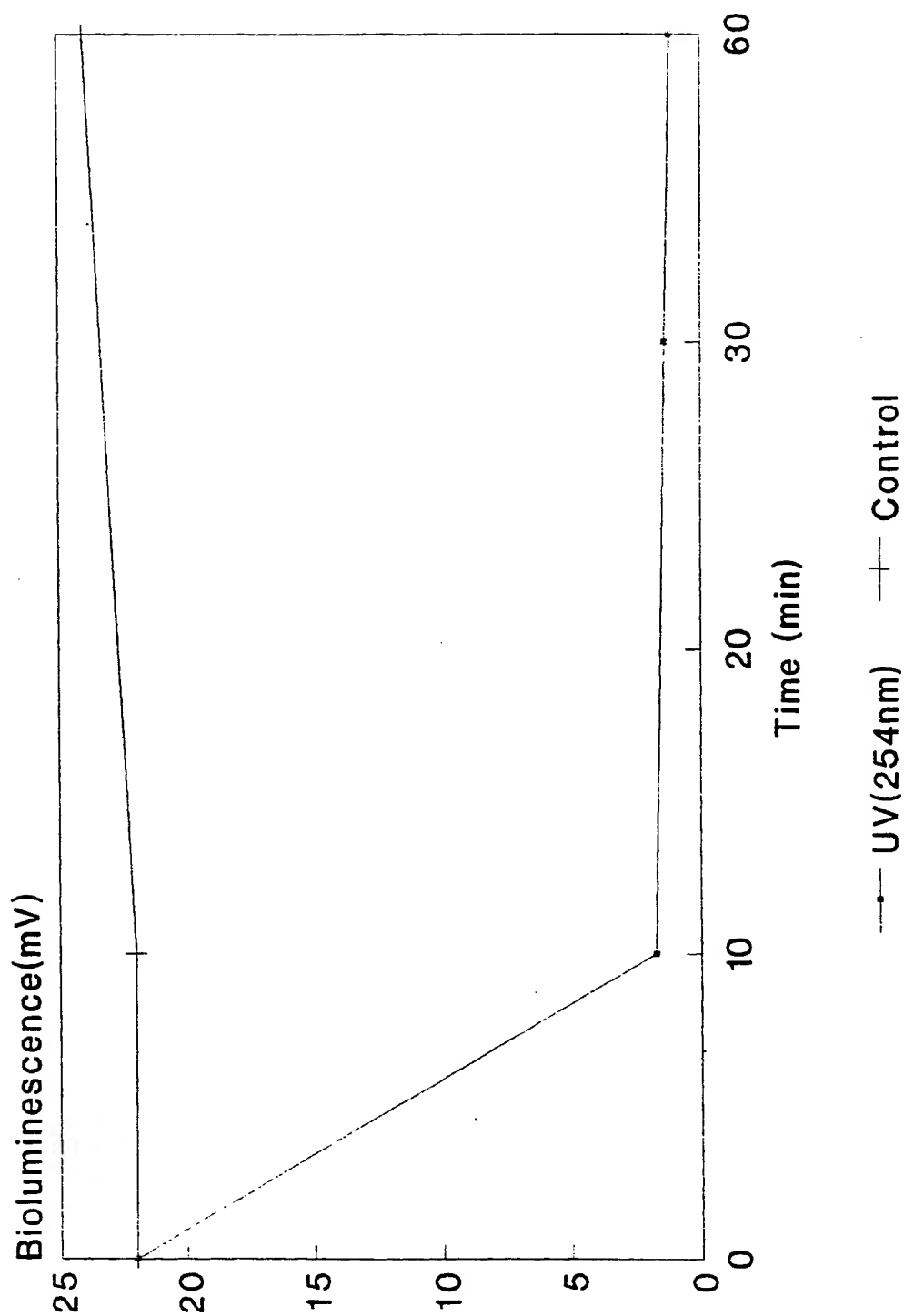


Figure 9f

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E. coli pCSS302/BW322
Antibiotic: Ofloxacin

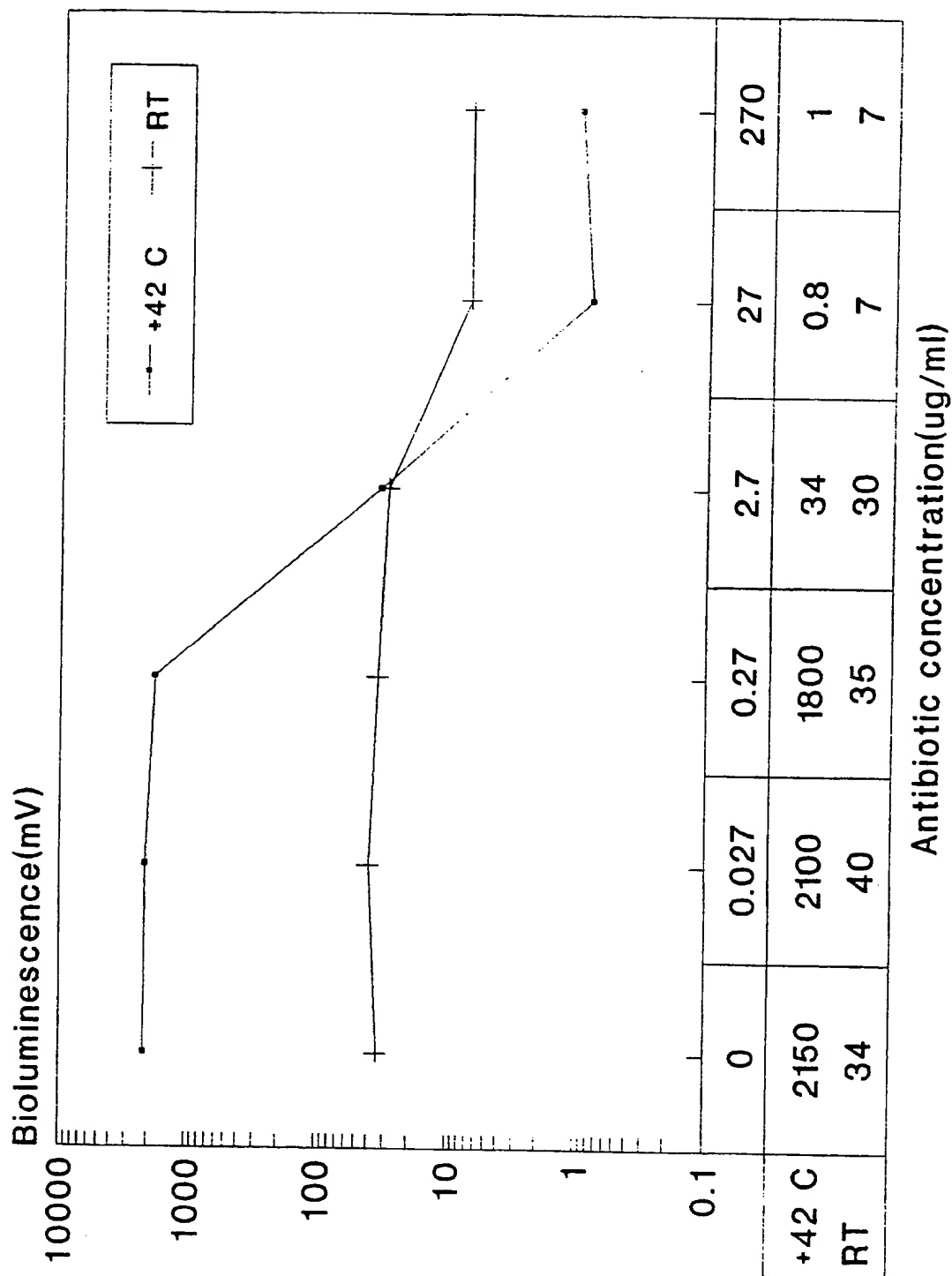


Figure 9g

E.coli pCSS302/BW322
Antibiotic:Ciprofloxacin

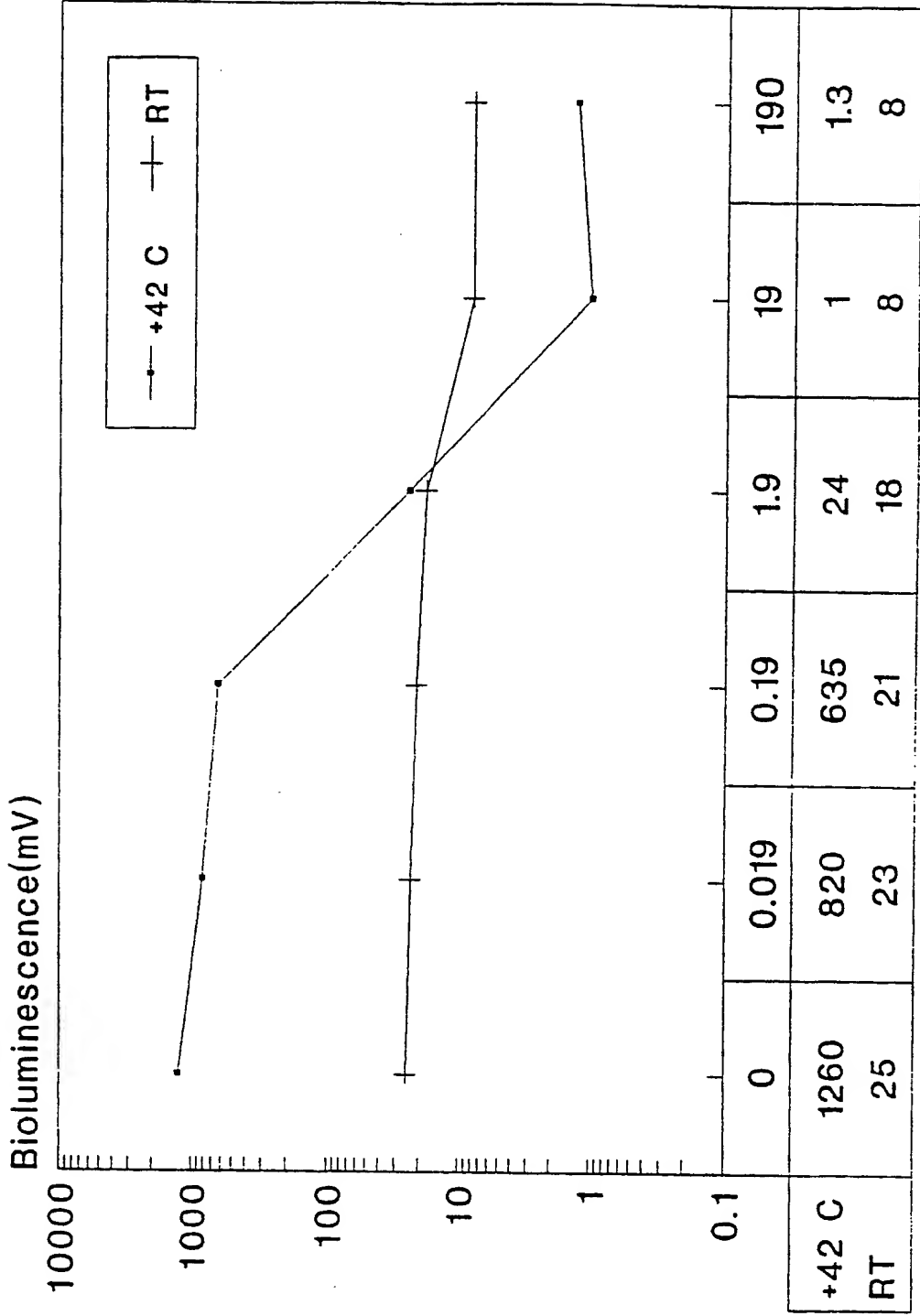


Figure 9h

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E. coli pCSS305/BW322
Antibiotic: Ciprofloxacin

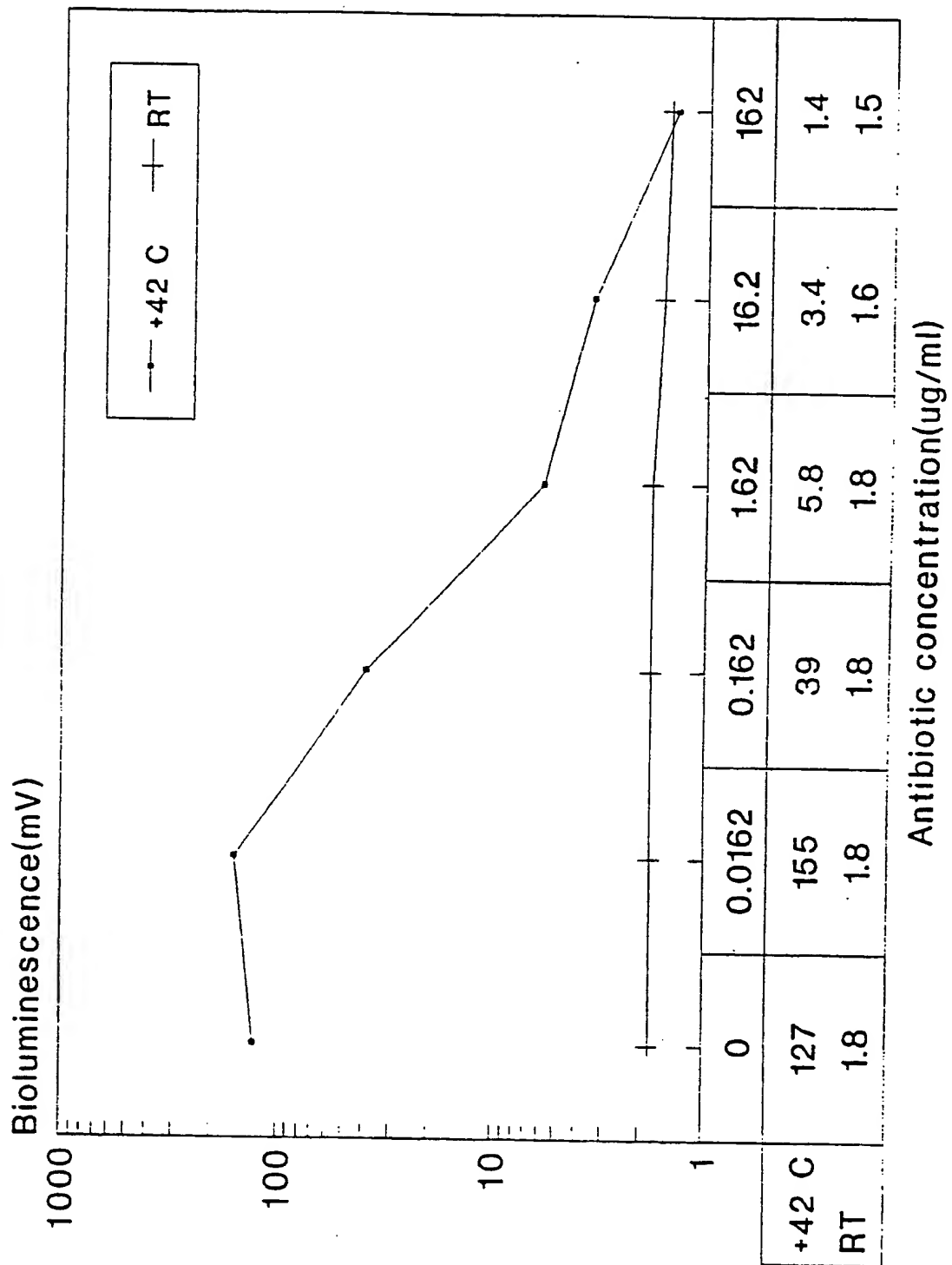


Figure 9i

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E. coli pCSS305/BW322
Antibiotic: Ofloxacin

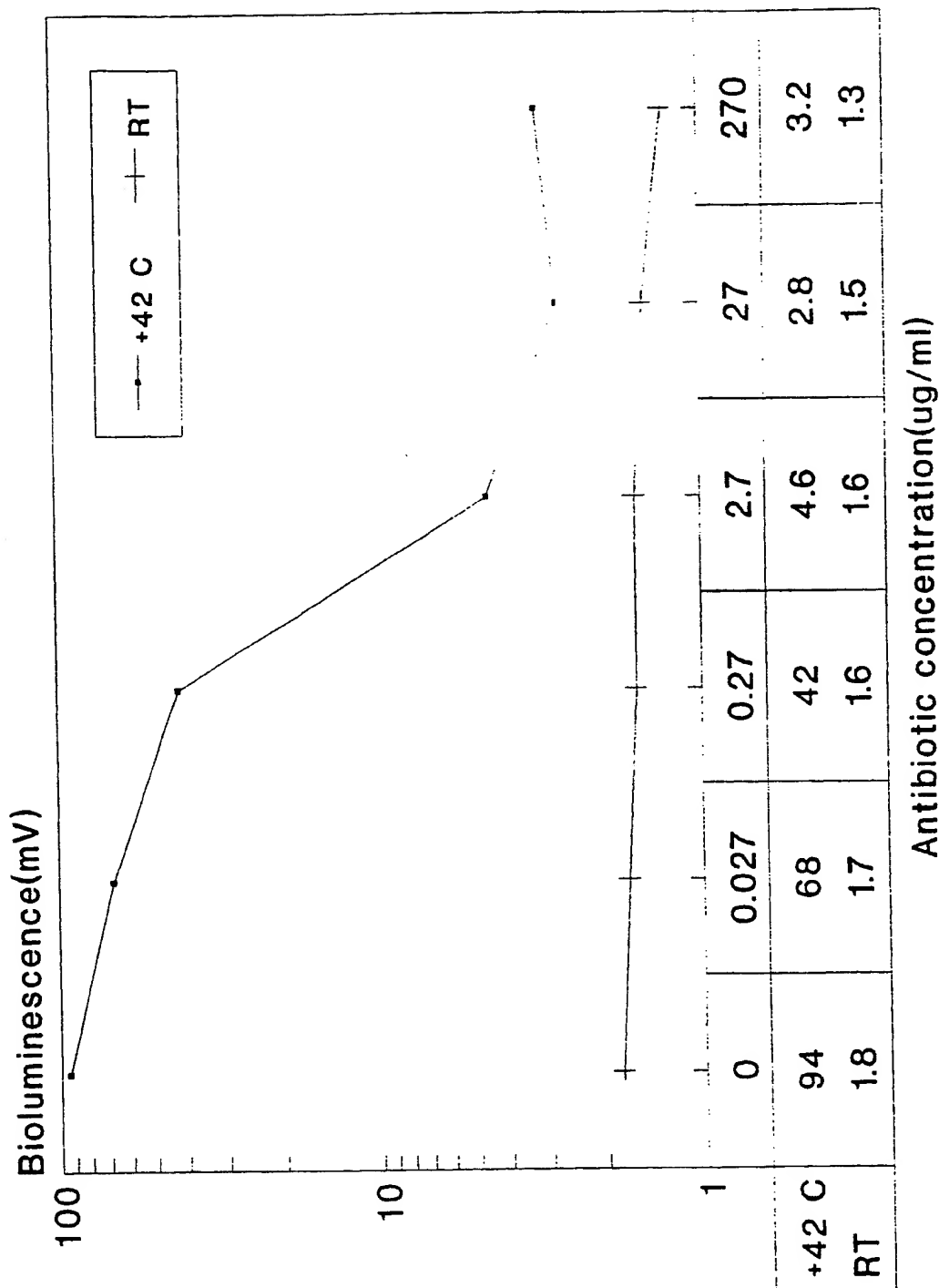
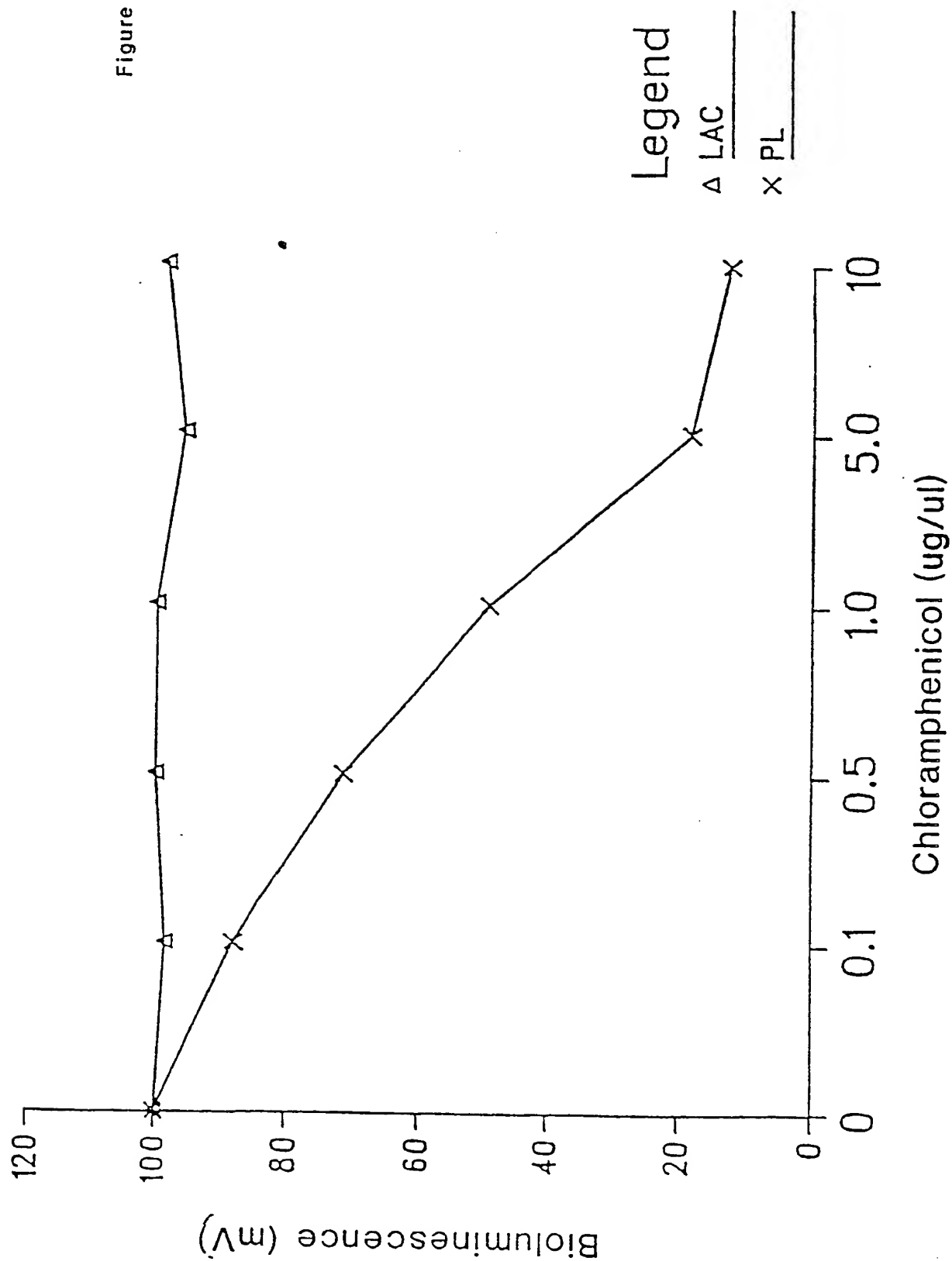


Figure 9j

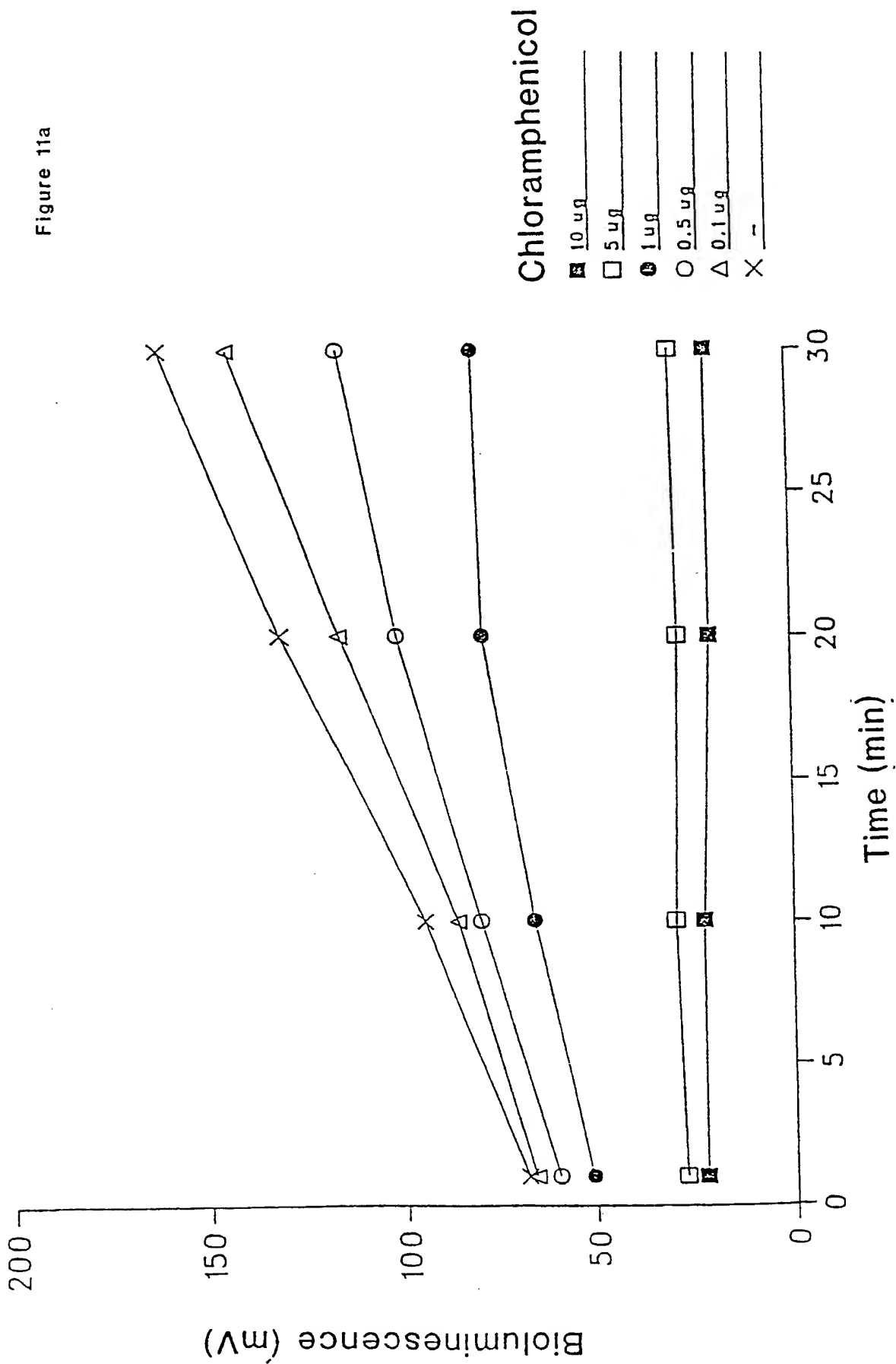
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Figure 10



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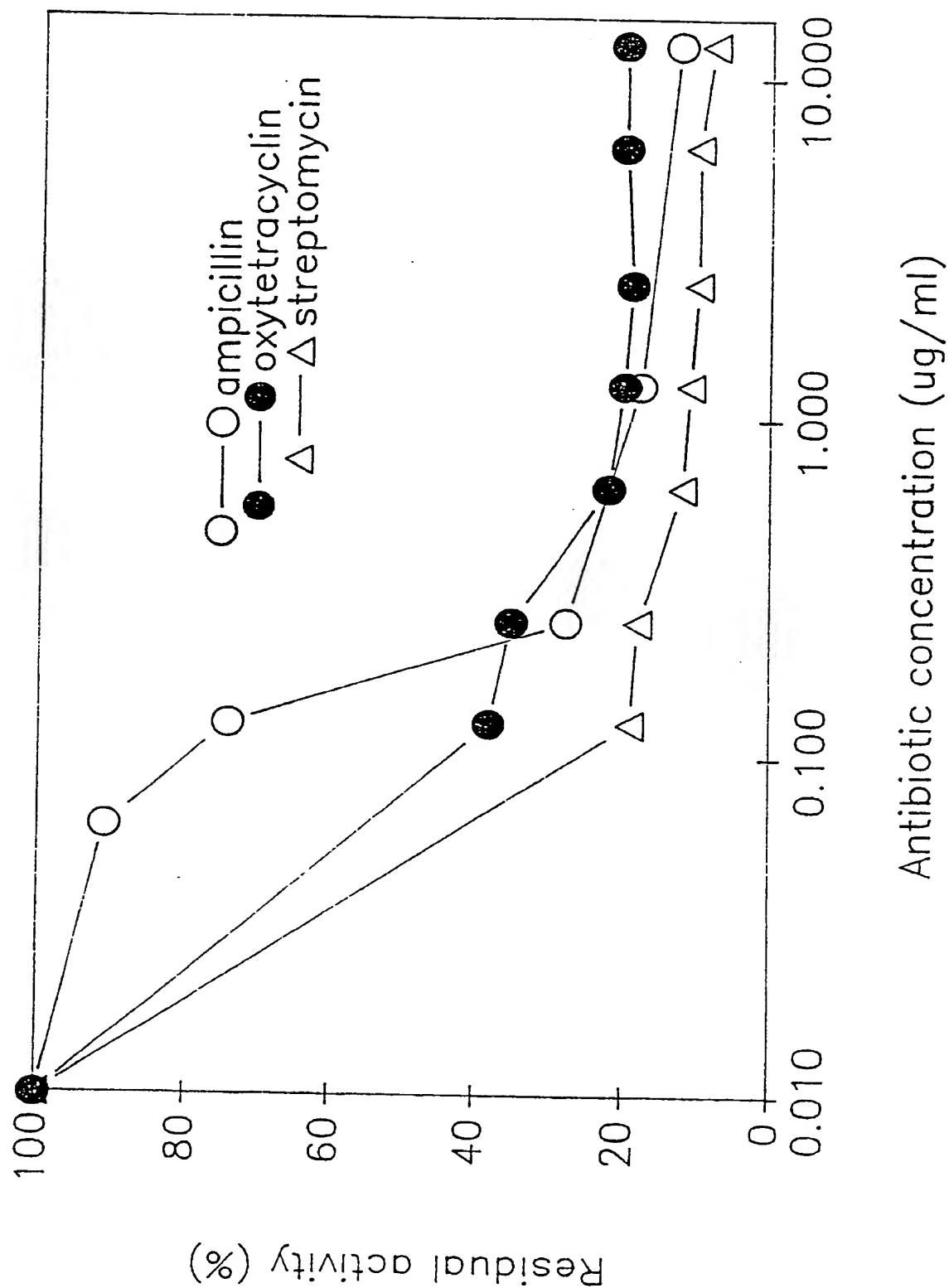
Figure 11a



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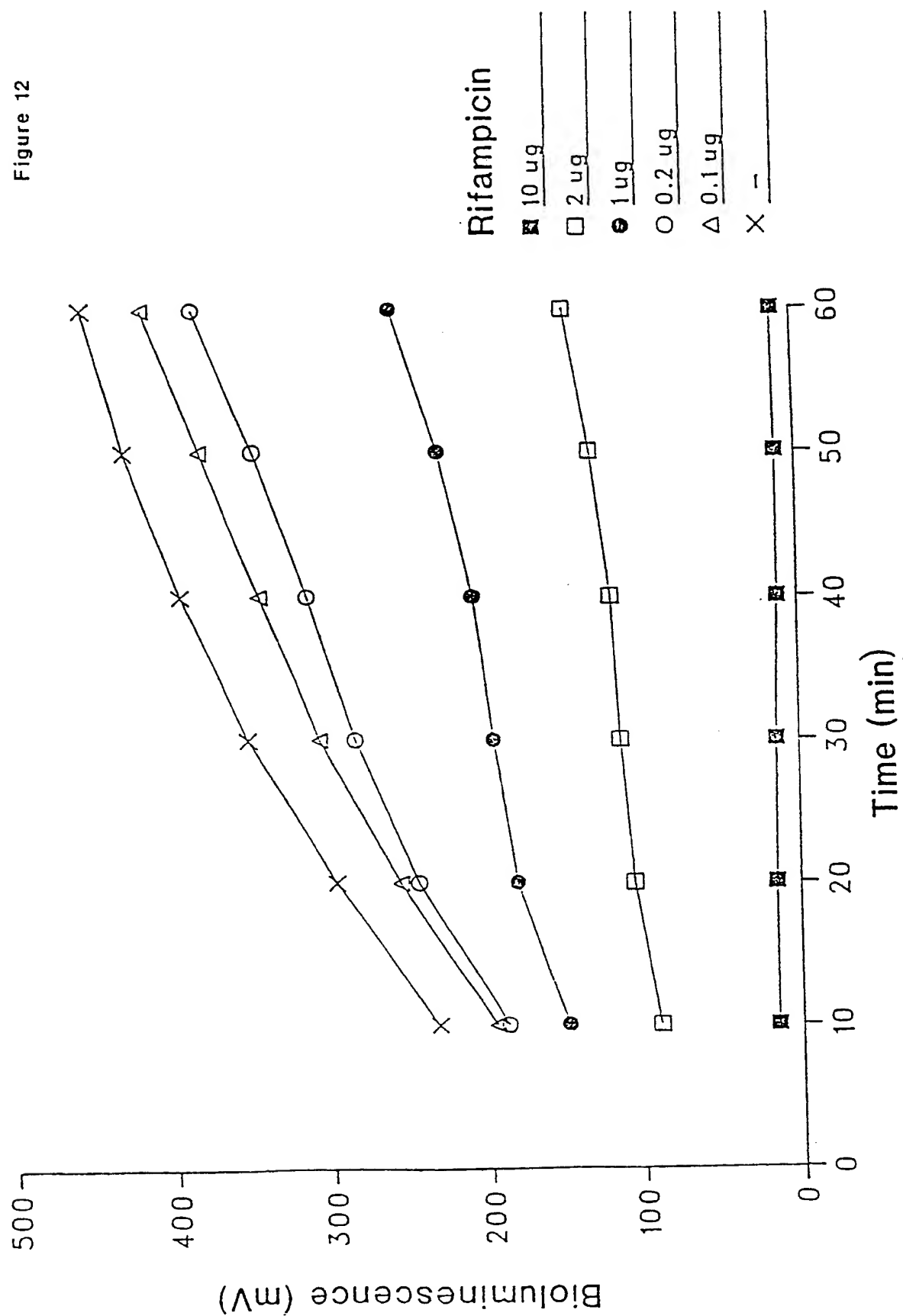
Figure 11b

Determination of antibiotics using *B. subtilis* and LucGR
under strong inducible promoter system



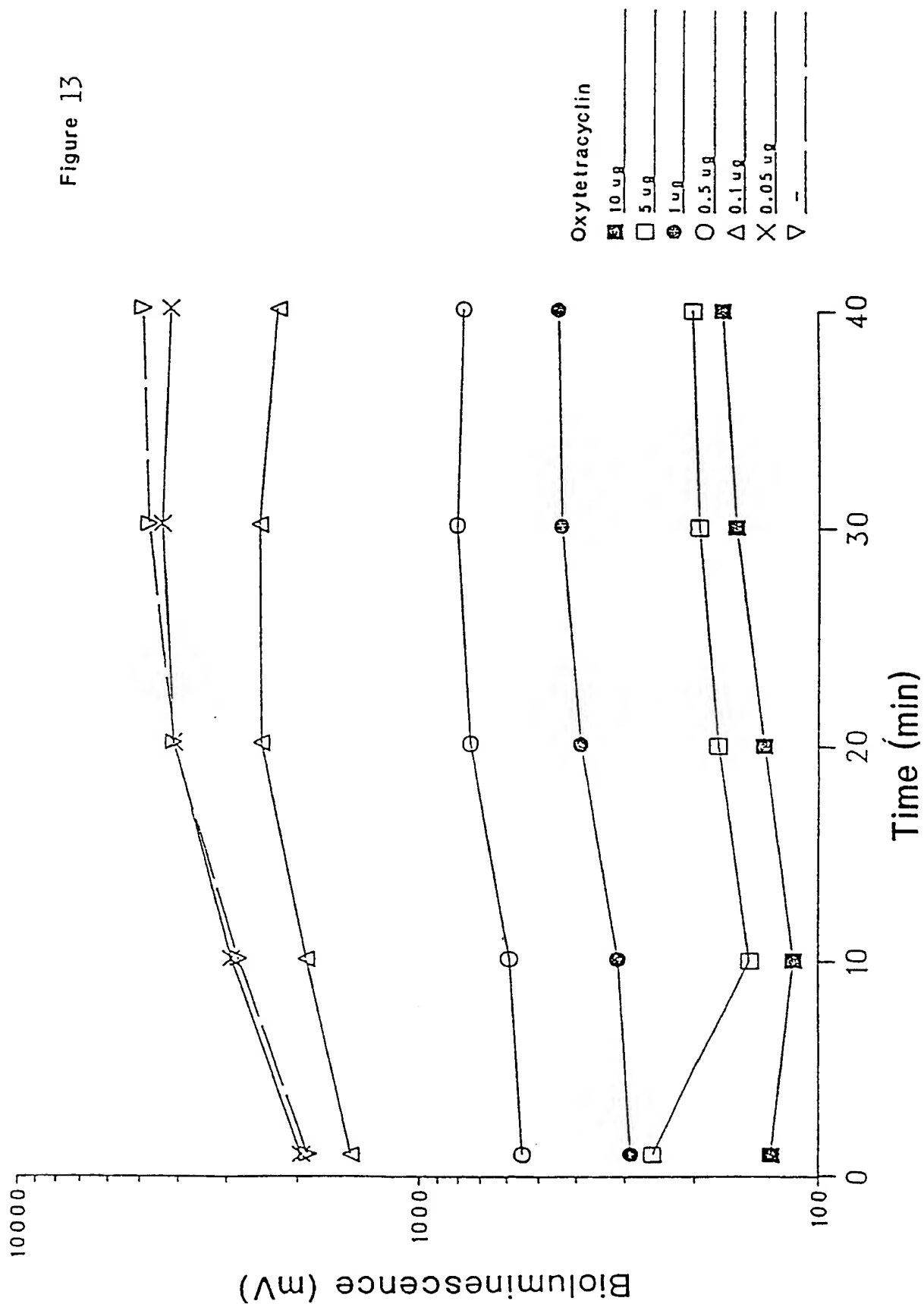
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Figure 12



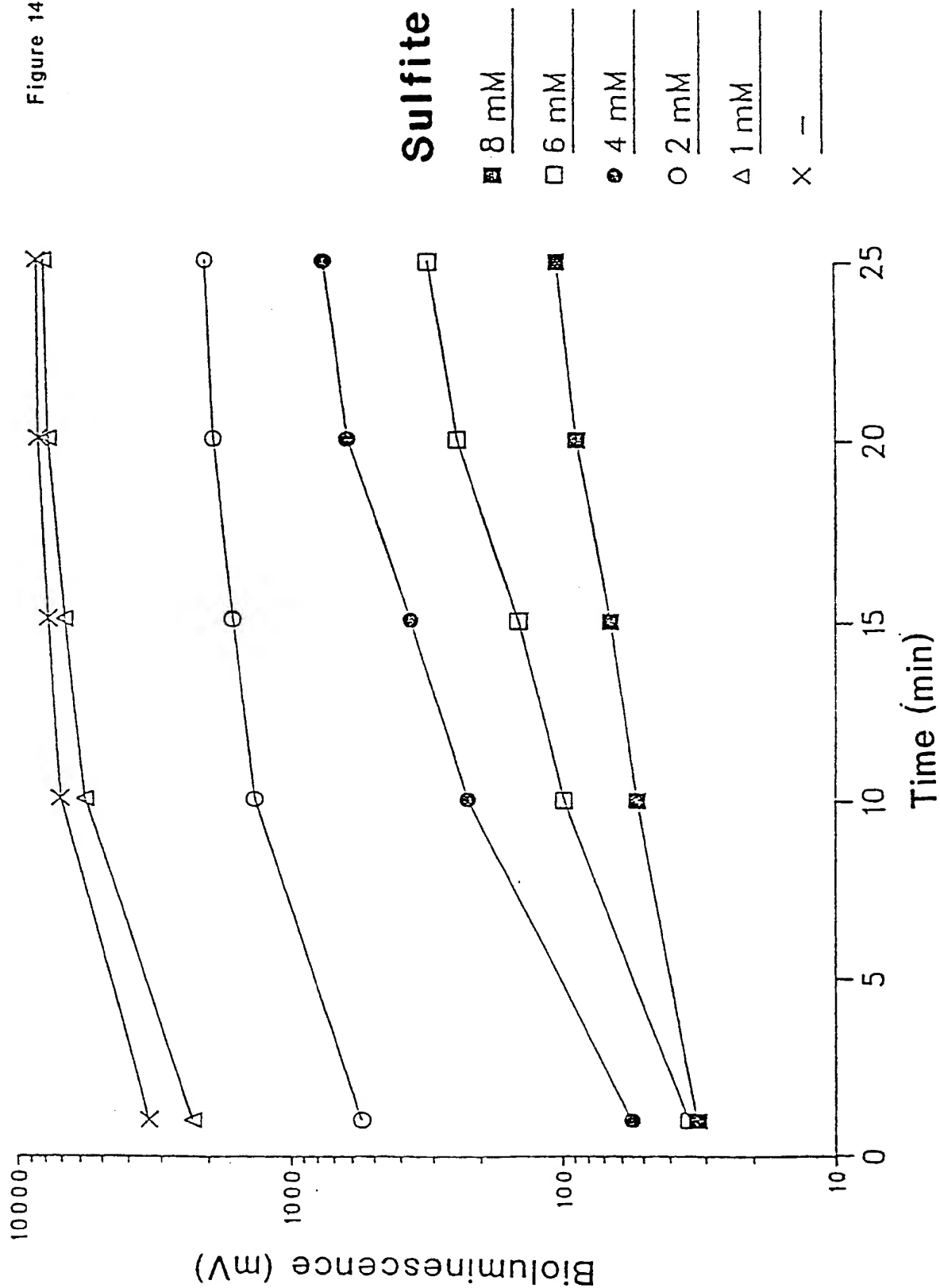
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Figure 13



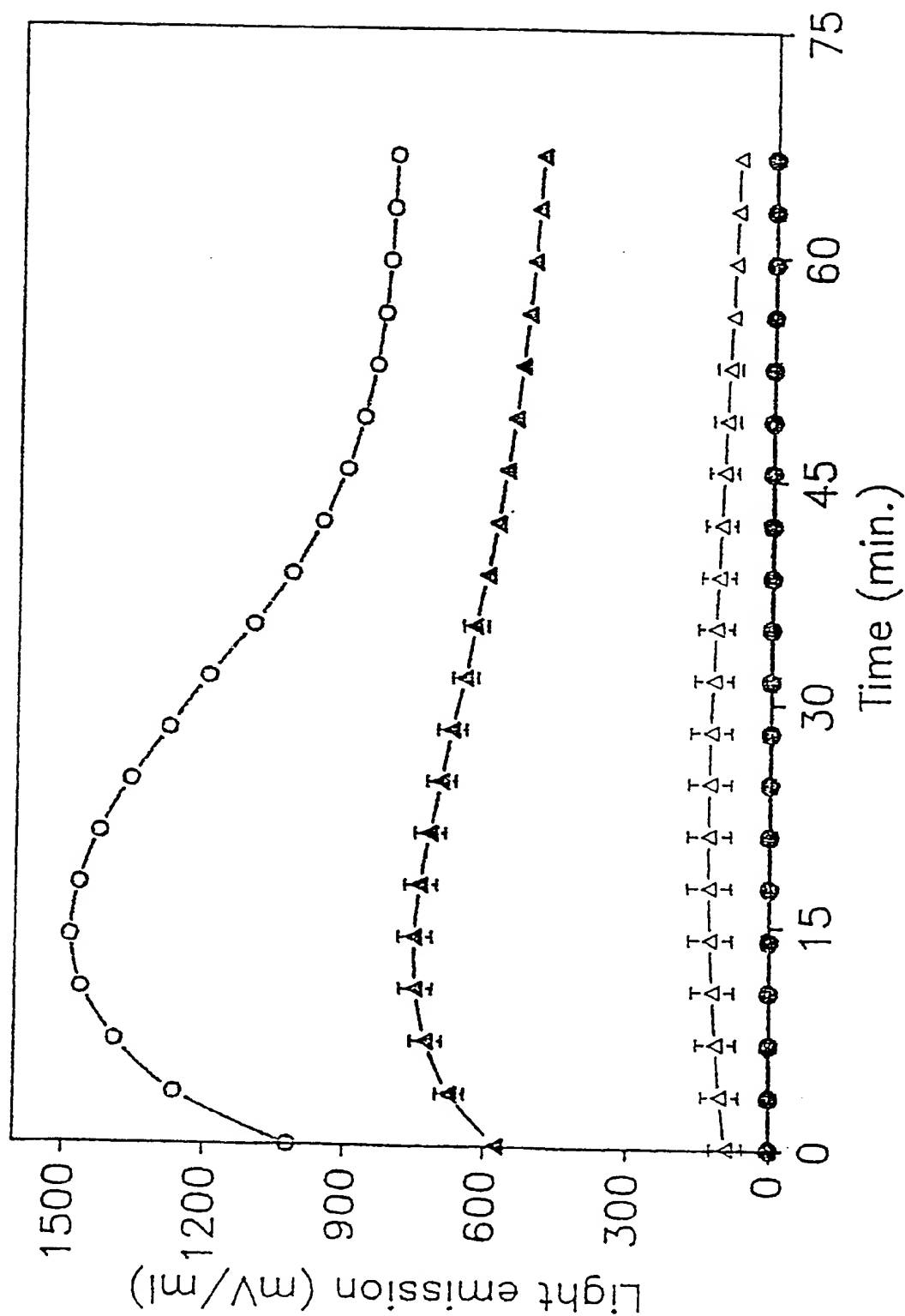
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Figure 14a



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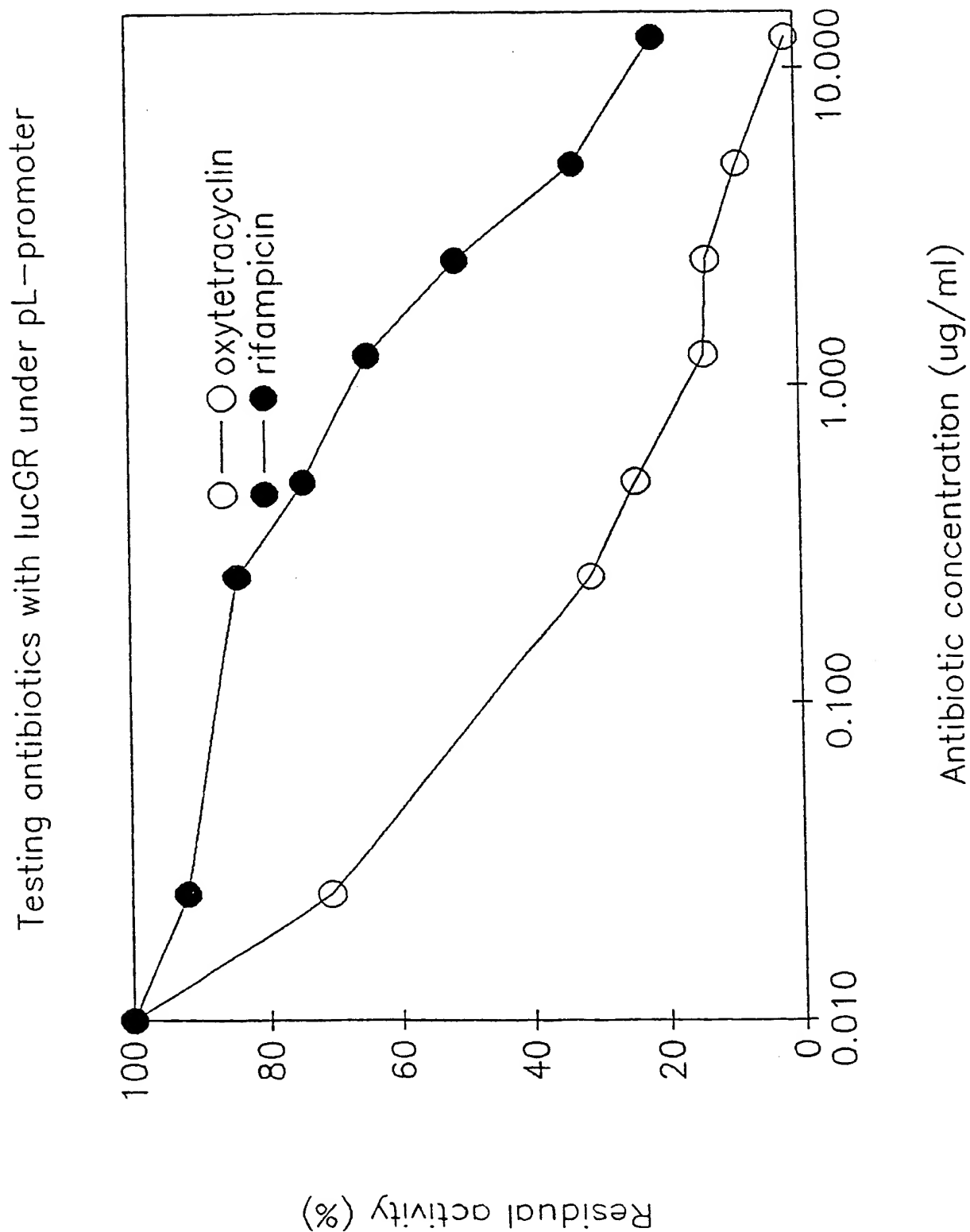
Figure 14b



○—○ no Cd, ▲—▲ 50.0 $\mu\text{g/ml}$ Cd, △—△ 100.0 $\mu\text{g/ml}$ Cd, ●—● 200.0 $\mu\text{g/ml}$ Cd

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Figure 15



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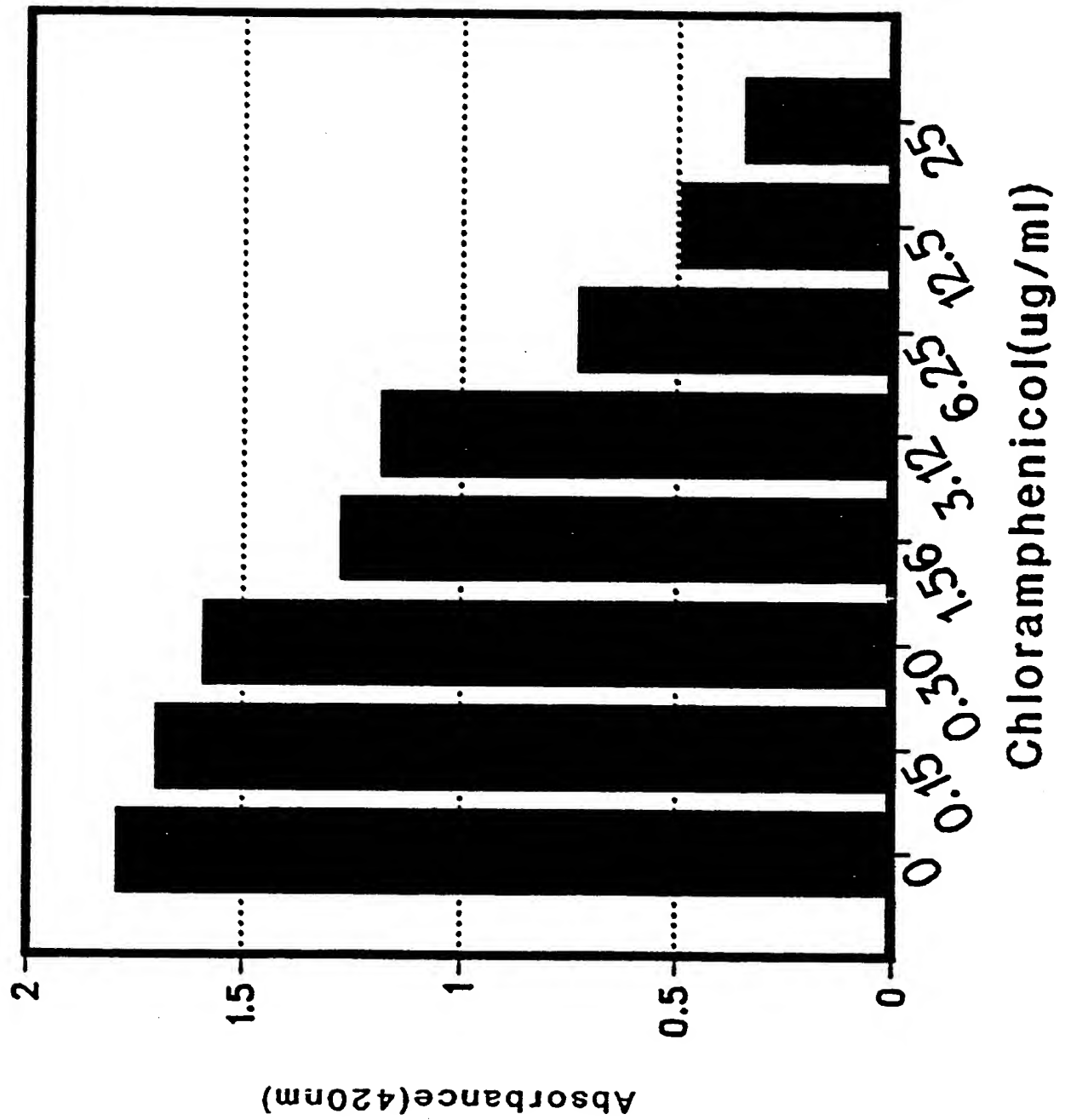
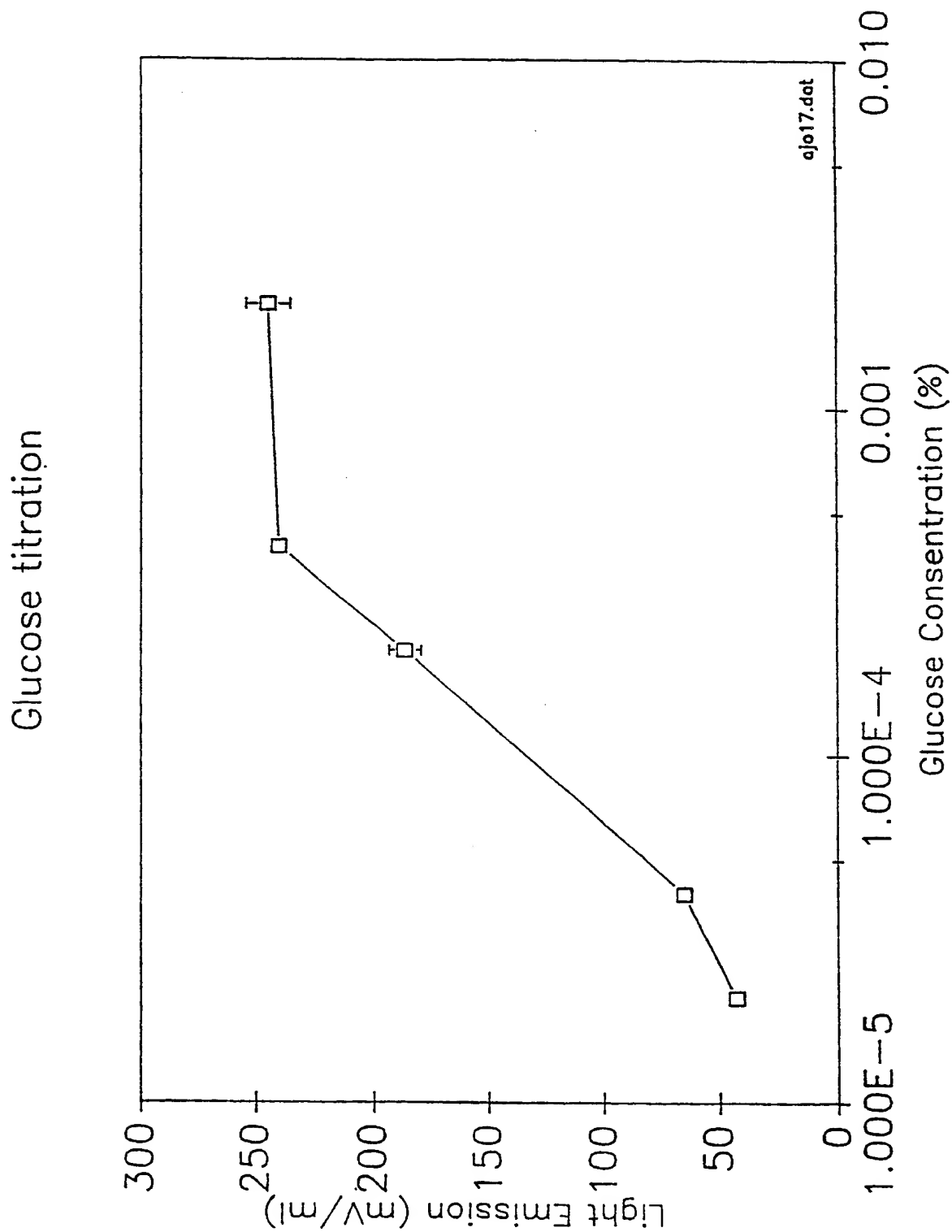


Figure 16

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Figure 17a



INTERNATIONAL SEARCH REPORT

International Application No PCT/FI 90/00112

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC IPC5: C 12 Q 1/02//C 12 Q 1/68, 1/66, C 12 N 15/69														
II. FIELDS SEARCHED <div style="text-align: center; margin-top: 5px;">Minimum Documentation Searched⁷</div> <table style="width: 100%; border: none;"> <tr> <td style="width: 25%; border: none; vertical-align: top;"> <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <th style="padding: 2px;">Classification System</th> <th style="padding: 2px;">Classification Symbols</th> </tr> <tr> <td style="padding: 5px; height: 40px; vertical-align: top;">IPC5</td> <td style="padding: 5px; vertical-align: top;">C 12 Q, C 12 N</td> </tr> </table> </td> <td style="border: none;"></td> </tr> </table> <div style="text-align: center; margin-top: 5px;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched⁸</div> <p style="margin-top: 10px;">SE,DK,FI,NO classes as above</p>			<table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <th style="padding: 2px;">Classification System</th> <th style="padding: 2px;">Classification Symbols</th> </tr> <tr> <td style="padding: 5px; height: 40px; vertical-align: top;">IPC5</td> <td style="padding: 5px; vertical-align: top;">C 12 Q, C 12 N</td> </tr> </table>	Classification System	Classification Symbols	IPC5	C 12 Q, C 12 N							
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<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>														
IV. CERTIFICATION <table style="width: 100%; border: none;"> <tr> <td style="width: 50%; border: none; vertical-align: top;"> <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <th style="padding: 2px;">Date of the Actual Completion of the International Search</th> </tr> <tr> <td style="padding: 5px;">17th July 1990</td> </tr> </table> <table border="1" style="width: 100%; border-collapse: collapse; margin-top: 5px;"> <tr> <th style="padding: 2px;">International Searching Authority</th> </tr> <tr> <td style="padding: 5px; text-align: center;">SWEDISH PATENT OFFICE</td> </tr> </table> </td> <td style="width: 50%; border: none; vertical-align: top;"> <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <th style="padding: 2px;">Date of Mailing of this International Search Report</th> </tr> <tr> <td style="padding: 5px; text-align: center;">1990-07-23</td> </tr> </table> <table border="1" style="width: 100%; border-collapse: collapse; margin-top: 5px;"> <tr> <th style="padding: 2px;">Signature of Authorized Officer</th> </tr> <tr> <td style="padding: 5px; text-align: center;"> Niklas Forslund </td> </tr> </table> </td> </tr> </table>			<table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <th style="padding: 2px;">Date of the Actual Completion of the International Search</th> </tr> <tr> <td style="padding: 5px;">17th July 1990</td> </tr> </table> <table border="1" style="width: 100%; border-collapse: collapse; margin-top: 5px;"> <tr> <th style="padding: 2px;">International Searching Authority</th> </tr> <tr> <td style="padding: 5px; text-align: center;">SWEDISH PATENT OFFICE</td> </tr> </table>	Date of the Actual Completion of the International Search	17th July 1990	International Searching Authority	SWEDISH PATENT OFFICE	<table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <th style="padding: 2px;">Date of Mailing of this International Search Report</th> </tr> <tr> <td style="padding: 5px; text-align: center;">1990-07-23</td> </tr> </table> <table border="1" style="width: 100%; border-collapse: collapse; margin-top: 5px;"> <tr> <th style="padding: 2px;">Signature of Authorized Officer</th> </tr> <tr> <td style="padding: 5px; text-align: center;"> Niklas Forslund </td> </tr> </table>	Date of Mailing of this International Search Report	1990-07-23	Signature of Authorized Officer	Niklas Forslund		
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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	EP, A1, 0121386 (CELLTECH LIMITED) 10 October 1984, see especially example 3 --	1-4,6,8- 11
A	SE, B, 424090 (TECHNION RESEARCH & DEVELOPMENT FOUNDATION LTD.) 28 June 1982, see the whole document --	19-22
A	US, A, 4806471 (SOREN MOLIN ET AL.) 21 February 1989, see the whole document -- -----	1-23

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. PCT/FI 90/00112

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the Swedish Patent Office EDP file on **osine**.
The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A1- 0281104	88-09-07	NONE	
EP-A1- 0121386	84-10-10	AU-B- 565352	87-09-10
		AU-D- 2812484	84-10-09
		DE-A- 3471688	88-07-07
		GB-A-B- 2136814	84-09-26
		JP-T- 60501837	85-10-31
		WO-A- 84/03713	84-09-27
SE-B- 424090	82-06-28	SE-A- 8104898	82-02-26
US-A- 4806471	89-02-21	AU-B- 561754	87-05-14
		AU-B- 569043	88-01-21
		AU-D- 1948783	84-04-04
		AU-D- 2033083	84-04-04
		CA-A- 1235667	88-04-26
		DE-A- 3377919	88-10-13
		EP-A-B- 0106542	84-04-25
		EP-A- 0109150	84-05-23
		US-A- 4760022	88-07-26
		WO-A- 84/01171	84-03-29
		WO-A- 84/01172	84-03-29

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